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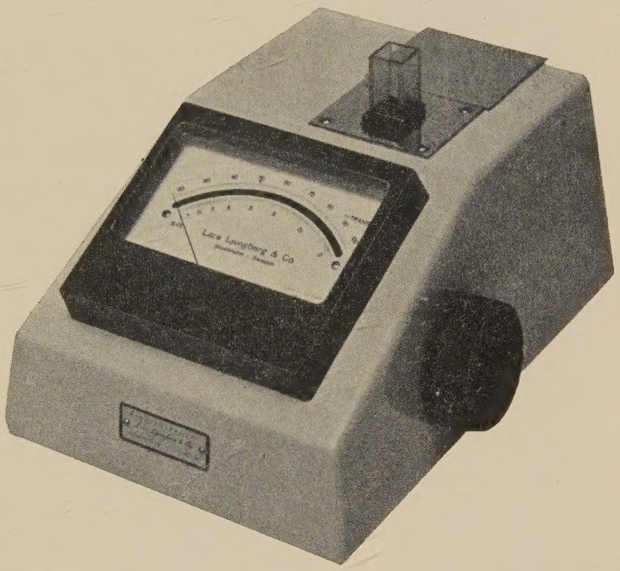

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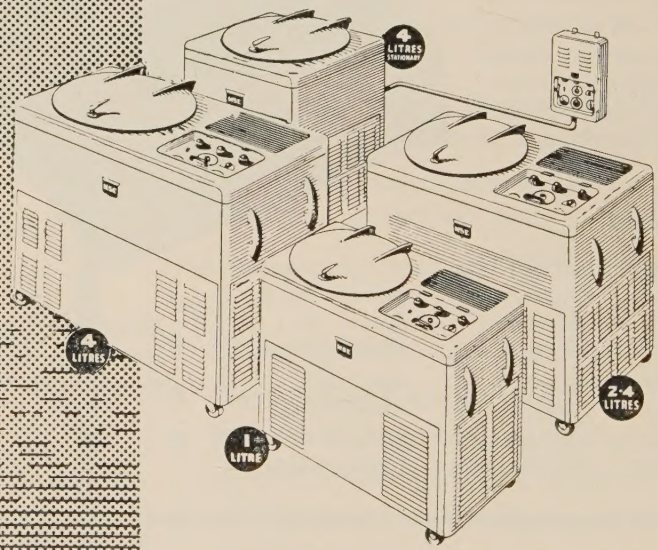

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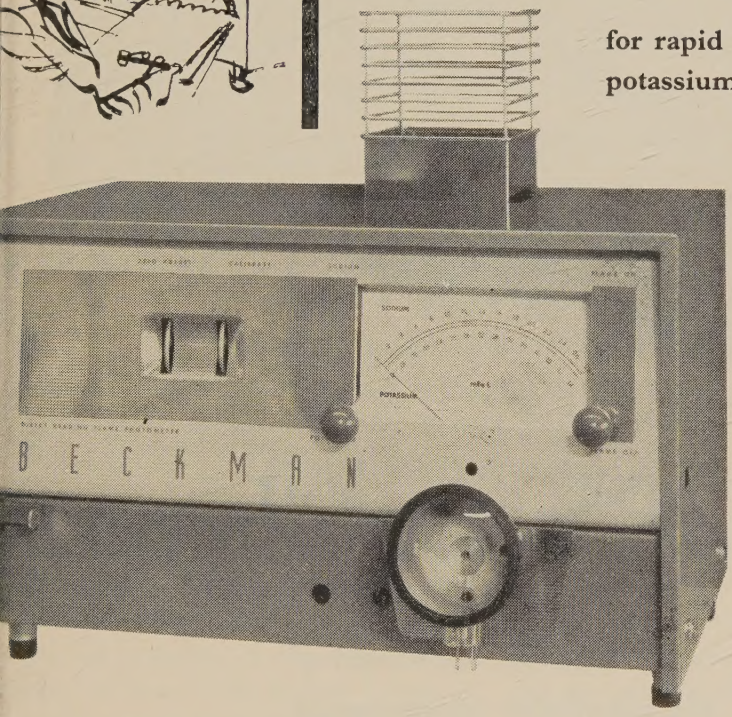

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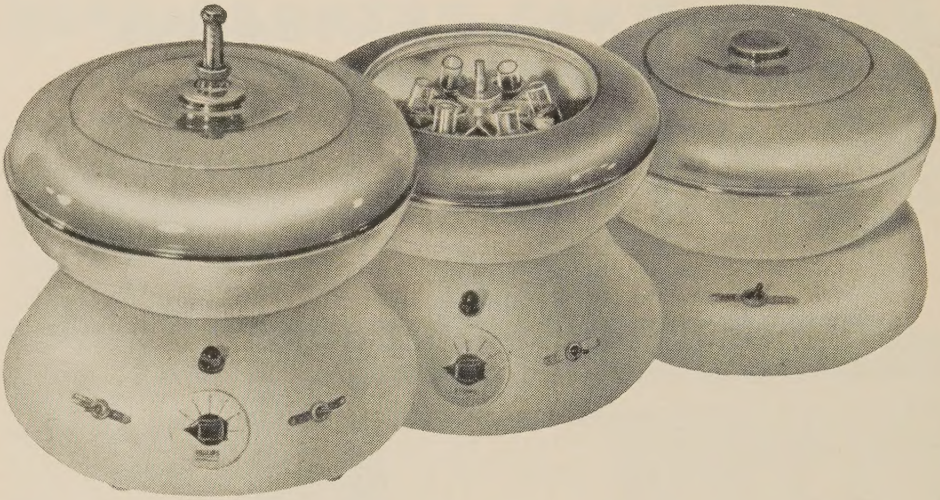

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ETERMINATION OF HISTAMINE IN BLOOD AND URINE BY ABSORPTION ON AMBERLITE IRC — 50

WITH SPECIAL REFERENCE TO THE NORMAL VALUES FOR HISTAMINE IN HUMAN BLOOD AND AN ANALYSIS OF THE SPECIFICITY OF THE METHOD

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(Received for publication February 12, 1958)

The first usable method for determination histamine in blood was published by Bar- m & Gaddum (1935). After a modifica- by Code (1937) this method has been d in the majority of investigations on amine in blood. The normal values for amine in whole blood in human subjects ained by this method vary between 2.5 9.0 μg per 100 ml (for references see reviews by Code, 1952, and Rocha de a, 1955). Recently a chemical method for amine determination in blood has been oduced by Lowry, Graham, Harris, Prie- Marks & Bregman (1954). The mean mal value for histamine in whole blood ained by this method was 8.2 μg per ml.

The highest concentration of histamine was nd in the white cells, which contain 70— per cent of all histamine extractable from whole blood (Code, 1937; Rose & Weil, 1939; Valentine & Lawrence, 1948; Graham, Lowry, Wheelwright, Leny & Parish, 1955). Small quantities of histamine have also been found in blood plasma (Code, 1937; Emmelin, 1945; McIntire, Roth & Sproull, 1950) but the question, whether it is present in a free (Emmelin, 1945) or physiologically inactive form (Colldahl, Holmberg & Laurell, 1946), remains unsettled.

A technically simple method for determination of histamine in urine, based on a principle devised by Bergström & Hansson (1951), was recently described by Dunér & Pernow (1956). The histamine was adsorbed on an Amberlite IRC-50 column, eluated with hydrochloric acid and quantitatively determined on isolated guinea-pig ileum. In the present investigation the same principle with slight modification is also used to estimate the amount of histamine in blood.

In healthy subjects the method gives reli-

able values for histamine in blood and urine. However, in pathological conditions other smooth muscle-stimulating substances may conceivably occur in blood and urine in amounts sufficiently large to influence the values. A study of the adsorption behavior of 5-hydroxytryptamine (5 HT), acetylcholine, substance P, and potassium on the Amberlite column as well as a quantitative comparison between the effect of these substances and of histamine on the guinea-pig ileum are therefore included in this paper. Finely the urinary excretion and assay of histamine were studied after administration of promethazine in man.

DETERMINATION OF HISTAMINE IN BLOOD

Method.

Blood samples were obtained from 25 healthy human subjects, males and females, aged 22—30 years. The samples were collected in the morning with the subjects fasting. The amount of histamine was determined in whole blood, blood cells and plasma. For whole blood 15 cc of venous blood was taken, immediately mixed with 100 cc of distilled water, and shaken in order to obtain complete hemolysis. Four volumes of acetone were immediately added, and after stirring and filtering the acetone was evaporated *in vacuo* on a boiling water-bath. The residue was adjusted to pH 6.5, and passed through an Amberlite IRC-50 column measuring 10 by 80 mm. After adsorption the column was acidified with 1N HCl and the elution was then continued with 0.01 N HCl. After neutralisation the extract was mixed with 10 volumes of a 1:1 alcohol-acetone solution and placed in th cold (—15°) for two hours. By this procedure mos of the salts were precipitated. After centrifugatio the clear centrifugate was evaporated *in vacu* until all alcohol and acetone had evaporated. Th extract was then immediately used for the bio logical assay, which was carried out on isolate guinea-pig ileum. Further details of the extractio and quantitative determination procedure are de scribed elsewhere (Dunér & Pernow, 1956).

In 17 cases 50 cc of blood was collected and im mediately centrifuged for 20 minutes to separat the blood cells from the plasma. The serum wa poured off, treated with 4 volumes of acetone an extracted in the same manner as described abov

The blood cells were shaken with 4 volumes o ether and ground in a meat mincer to obtain complete lysis, 2 cc of distilled water was nex added and the ether was evaporated *in vacuo* on boiling water-bath. The extraction was then con tinued in the same manner as for the whole bloo and serum samples.

In the urine about two-thirds of the total amou of histamine is present in an inactive form. Th histamine can not be extracted and assayed unt after hydrolysis. To find out whether this appli to blood as well, hydrolysis of blood samples wa done in some cases. The procedure was the sam as that described for urine (Dunér & Perno 1956).

Results.

The content of histamine in serum ranged fro 0.6—2.8 μg per 100 ml of blood (mean: 1.29 ± 0.17 The corresponding value for blood cells was 1.0 4.6 μg per 100 ml of blood (mean: 2.66 ± 0.33 In whole blood the range of the histamine conte was 1.5—8.8 μg per 100 ml of blood (mea 4.46 ± 0.37). In hydrolysed whole blood the co responding values were 2.1—8.3 μg per 100 ml blood (mean: 4.15 ± 9.39), respectively (Table I

Table I. *Histamine in blood (μg per 100 ml of whole blood)*

	Blood serum	Blood corpuscles	Whole blood nonhydrolyzed	Whole blood hydrolyzed	Number of subjects
Mean	1.29 ± 0.17	2.66 ± 0.33	4.46 ± 0.37	4.15 ± 0.39	25
Limits	0.6 — 2.8	1.0 — 4.6	1.5 — 8.8	2.1 — 8.3	

IDENTIFICATION OF HISTAMINE IN MIXTURES OF DIFFERENT SMOOTH MUSCLE-STIMULATING AGENTS

Method.

n each experiment histamine was mixed in ne solution with one of the following substances: IT, acetylcholine, substance P or potassium, and solution was then passed through the column. e method for adsorption and elution was the ne as that described for the blood. The eluate s collected in different fractions, which were arately assayed on the guinea-pig ileum after tralisation. The total activity was first compa- to a histamine phosphate standard and expres- in terms of histamine base. The assay was then eated after addition of dihydroergotamine, atro- e or an antihistaminic (Lergitin, Recip) to the rode's solution. The activity was expressed in tamine or units of substance P, the latter after lition of Lergitin to the bath-fluid. In each ex- iment the ratio of activity between histamine l the other substance was determined.

Results.

Histamine + 5 HT. Both histamine and IT were adsorbed on the Amberlite and tained in the same fractions after elution th hydrochloric acid. It was therefore pos- le to separate them only by assaying the ivity of the fractions before and after ad- ion of dehydroergotamine (DHE) and rgitin. The ratio of activity of histamine 5HT on the isolated guinea-pig ileum ried between 5:1 to 20:1. Two typical periments are presented in Table II.

Histamine + acethylcholine. Like hista- ne and 5HT, acetylcholine was completely sorbed on the ion exchanger and was eluted the same fractions as histamine. Separa- n of the effect of the two substances was ne after adding atropine or Lergitin to bath-fluid. The ratio of activity of hista-

Table II. *Recovery of histamine and 5HT after adsorption on Amberlite IRC-50.*

A. Original solution: 6 μg histamine + 40 μg 5HT in 100 ml of saline solution. Ratio of actvity hi: 5HT 13: 1.

Eluate fraction No.	Volume in ml	pH	Total activity in terms of histamine (μg)	Activity after DHE in terms of histamine (μg)
1	100	6.5	0	0
2	48	6.0	0	0
3	3	5.8	1.2	0
4	2	2.0	6.5	5.0
5	3	1.5	1.3	0.9
			Yield 9.0	5.9

B. Original solution: 6 μg histamine + 10 μg 5HT in 100 ml of saline solution. Hi: 5HT = 20: 1.

Eluate fraction No.	Volume in ml	pH	Total activity in terms of histamine (μg)	Activity after DHE in terms of histamine (μg)
1	100	6.5	0	0
2	50	6.0	0	0
3	9.8	3.2	0	0
4	1.8	2.1	4.8	4.8
5	2.0	1.2	1.3	1.0
			Yield 6.3	5.8

mine to acethylcholine was 1:0.15—1:0.8. A typical experiment is shown in Table III.

Histamine + substance P. In contrast to all the above-mentioned drugs, substance P was not adsorbed on the Amberlite and was therefore found in the original solution when passed through the column. The activity of substance P in the eluate was, thus, readily separated from that of histamine. The ratio of activity histamine: substance P varied between 8.6:1 and 11:1 (μg: unit), (see Table III).

Table III. *Recovery of histamine, acetylcholine and substance P after adsorption on Amberlite IRC-50.*

A. Original solution: 20 μg of histamine + 20 μg of acetylcholine in 100 ml of saline solution. Ratio of activity hi: ach 1: 0.15.

Eluate fraction No.	Volume	pH	Total activity in terms of histamine (μg)	Activity after atropine in terms of histamine (μg)
1	100	6.5	0	0
2	50	6.3	0	0
3	6	5.3	0	0
4	2.0	2.0	66.5	13.0
5	2.5	1.4	31.2	4.8
6	2.1	1.3	6.0	0
			Yield 103.7	17.8

B. Original solution: 20 μg of histamine + 75 units of substance P in 50 ml saline solution after Lergitin in units of P. μg hi: unit P = 11: 1.

Eluate fraction No.	Volume	pH	Total activity in terms of histamine (μg)	Substance P activity in units
1	53	6.5	6.5	70
2	8.5	5.5	0.9	7
3	2.0	2.1	17.4	0
4	1.8	1.4	1.4	0
			Yield 26.2	77

Histamine + potassium. Varying amounts of histamine were mixed with potassium in quantities that normally occur in blood, *i. e.* 65—85 meq. per litre. The potassium was determined by flame photometry, but the different fractions were also assayed on the guinea-pig ileum. Potassium was adsorbed on the Amberlite and immediately eluted after addition of hydrochloric acid. Potassium was therefore obtained in the same fractions as histamine (Table IV).

Table IV. *Recovery of histamine and potassium after adsorption on Amberlite IRC-50*

The original solution contained 20 μ of histamine and 60 mg of potassium.

Eluate fraction No.	Volume	pH	Potassium in mg	Histamine in μg
1	20	6.5	1.6	0
2	25	6.5	0.3	0
3	5	5.6	0.4	0
4	2.1	1.9	39.3	14.3
5	2.3	1.3	17.3	4.1
6	4.0	1.2	6.4	0
			Yield 65.3	18.4

DETERMINATION OF HISTAMINE IN URINE CONTAINING PROMETHAZINE

A special problem arises when assaying the histamine content in blood or urine from patients receiving promethazine preparations. It has been shown that this drug is partly excreted in the urine in a free form after oral as well as after parenteral administration (Perlman, 1949). The presence of promethazine and other antihistaminics makes the biological assay of histamine impossible because of a successively decreasing sensitivity of the gut to histamine.

The method for extraction of histamine from blood and urine described here can however, after some modification be used during promethazine medication. It is then necessary to remove the antihistamine from the extracts prior to the biological assay. Most of the promethazine passes the column but some is also adsorbed and present in the histamine fraction obtained after eluation with hydrochloric acid. In order to separate the promethazine preparation (Lergigan Recip) from the histamine fractions we used

following method, which is based on the ervation of Rhone-Poulenc (1954) that methazine can be extracted by ether from lt solution. The eluates were neutralized h NaOH. Twenty volumes of peroxide- ether was added and the extract vigor- ly shaken. The ether was poured off and residue was heated on a waterbath at ut 60° C until all the ether had evapo- ed. Samples of all fractions were tested for methazine by adding equal amounts of centrated sulphuric acid, which in the pre- ce of promethazine gives a carmine red or (Rhone-Poulenc, 1954; Christiansson, 6). This test is very sensitive, giving a ectable color even in the presence of less n 1 μg of promethazine per ml.

RINARY EXCRETION OF HISTAMINE AFTER ORAL ADMINISTRATION OF PROMETHAZINE

n this study 50 mg promethazine was en intramuscularly in a single dose to five lthy subjects. The amount of free hista- e was determined in the 24-hour urine ring two days before and two days fol- ring administration of promethazine. For ee days the patients did not receive any er treatment. The results are collected in ble V, from which it will be seen that no nificant difference was found in the ount of histamine excreted during this dication. The figures obtained are within same limits as those previously found in lthy human subjects (Dunér & Pernow, 6).

Table V. *Urinary excretion of free histamine after promethazine administration in normal subjects*

Day	Treatment	Histamine in urine (μg per 24 hours)				
		Case 1	2	3	4	5
1	None	12	18	9.5	14	6.7
2	None	15	16	6.3	18	13
3	50 mg Lergitin	13	16	12	13	15
4	None	10	17	13	15	8.8

DISCUSSION

The normal values for histamine in whole od obtained by the method described here in good agreement with those reported by others using the method of Barsoum & Gaddum (1935). About one-thirds of the histamine content in whole blood was found in the serum which is inconsistent with some earlier investigations, for instance those by Code (1937) and Haworth & Mac Donald (1937) who did not find any histamine in plasma. However, from the values obtained by this method it cannot be concluded whether or not the histamine occurs in plasma in a biologically active form, because the extraction includes procedures which might liberate histamine, previously not occurring in free form. On the other hand, there is nothing in the blood corresponding to the conjugated histamine in urine, since no further increase in the amount of histamine is obtained in blood after hydrolysis (see Table I).

The urine normally contains several smooth muscle-stimulating substances besides histamine. Using a biological method for determination of histamine, based on its smooth muscle-stimulating properties, one main difficulty is to prove the specificity of the results of the assay. Among smooth muscle-stimulating principles extracted from urine, the following seem to be the most important: histamine, 5HT, kallidin (Werle, Kehl & Koebke, 1950), substance U (Beraldo, 1952), a specific biologically active

polypeptide found by Gomes (1955) but not yet given a name, and potassium. Since kallidin, substance U, and Gomes' principle were not available, we studied the behavior of another smooth muscle-stimulating polypeptide, substance P (Euler & Gaddum, 1931; Pernow, 1953, and others), which has not yet been extracted from urine. It is likely, though it has not been proved, that this substance behaves on the Amberlite in a manner similar to the above-mentioned polypeptides. It will be seen from Table III that substance P is not adsorbed on the Amberlite column and, hence, it has not interfered with the histamine assay.

Of the substances investigated here only potassium and 5HT were shown to be likely to interfere with the histamine assay. Both occur in blood and urine in sufficiently large amounts and both are adsorbed in the same manner as histamine on the Amberlite column. The influence of potassium is, however, eliminated by treating the extract with alcohol and acetone, which precipitates most of the salts.

5HT normally occurs in blood in amounts of 0.02—0.3 μg per ml (Erspamer, 1954; Sjoerdsma, Weissbach & Udenfriend, 1956, and others). The amount of 5HT demonstrated in urine varies from less than 50 μg per 24 hours (Udenfriend, 1955) up to 0.7 μg per ml (Lembeck & Neuhold, 1955). However, 5HT has only about one-tenth of the stimulating effect of corresponding amounts of histamine on the guinea-pig ileum and does therefore not normally interfere with the histamine assay. Yet, in rare cases with increased amounts of 5HT in blood and urine, as in carcinoidosis (Pernow & Waldenström, 1954), the content of 5HT might simulate a high histamine value. Th 5HT effect on the gut can, however, b completely blocked by addition of DHI (5 μg per ml) to the bath fluid, which is t be recommended when using this method o extraction and assay of histamine.

If an antihistaminic, for instance prome thazine, is administered to a subject, th method for extraction of histamine in bloo and urine has to be completed by a specia technique for removing the antihistamine be fore the biological assay. The promethazin medication did not influence the urinary ex cretion of free histamine, which is in con formity with the general conception tha antihistaminics do not interfere with the re lease of histamine.

SUMMARY

1. On the basis of a previously describe method for extraction of histamine fron urine, a modified technique for deter mination of histamine in blood is de scribed. The content of histamine i serum ranged from 0.6—2.8 μg per 10 ml of blood (mean 1.29 ± 0.17). Th corresponding values for blood cells wer 1.0—4.6, with a mean of 2.66 ± 0.33 μ per 100 ml of blood.
2. The specificity of the method was stu died by passing mixtures of histamin and other smooth muscle-stimulatin agents such as acetylcholine, 5-hydroxy tryptamine (5HT), substance P an potassium through the Amberlite co lumn. At the subsequent biological assa of the eluates, 5HT and potassium wer found in the same fractions as histamin
3. Because of this finding the earlier de scribed method for extraction and de

termination of histamine (Dunér & Pernow, 1956) was modified by a treatment of the eluates with alcohol-acetone in order to precipitate the salts, and by addition of dihydroergotamine to the bath-fluid in order to make the intestine insensitive to 5HT.

Administration of an antihistaminic, promethazine, renders the biological assay of histamine impossible because of excretion of promethazine in the urine. However, the promethazine can be eliminated by extraction with ether (Rhone-Poulenc, 1954). The excretion of histamine in the urine was found not to be influenced by oral administration of promethazine.

REFERENCES

soum, G. S. & Gaddum, J. H.: The pharmacological estimation of adenosine and histamine in blood. J. Physiol. *85,* 1, 1935.

aldo, W. T.: Substance U — a depressor and smooth muscle stimulating principle present in urine. Am. J. Physiol. *177,* 371, 1952.

gström, S. & Hansson, G.: The use of Amberlite IRC-50 for the purification of adrenaline and histamine. Acta Physiol. Scandinav. *22,* 87, 1951.

stiansson, G.: Påvisande av antihistaminer i kroppsvätskor. Nord. Med. *56,* 1751, 1956.

e, C. F.: The quantitative estimation of histamine in the blood. J. Physiol. *89,* 257, 1937.

e, C. F.: Histamine in blood. Physiol. Rev. *32,* 47, 1952.

dahl, H., Holmberg, C. C. & Laurell, C. B.: In what form does Histamine occur in plasma? Acta Physiol. Scandinav. *12,* 1, 1946.

ér, H. & Pernow, B.: Urinary excretion of histamine in healthy human subjects. Scandinav. J. Clin. Lab. Investigation *8,* 296, 1956.

nelin, N.: On the presence of histamine in plasma in a physiologically active form. Acta Physiol. Scandinav. *11,* Suppl. *34,* 1945.

Erspamer, V.: Pharmacology of indolealkylamines. Pharmacol. Rev. *6,* 425, 1954.

Euler, U. S. v. & Gaddum, J. H.: An unidentified depressor substance in certain tissue extracts. J. Physiol. *72,* 74, 1931.

Gomes, F. P.: A slow contracting substance in normal human urine. Brit. J. Pharmacol. *10,* 200, 1955.

Graham, H. T., Lowry, O. H., Wheelwright, F., Lenz, M. A. & Parish, H. H.: Distribution of histamine among leukocytes and platelets. Blood. *10,* 467, 1955.

Hawort, E. & Mac Donald, A. D.: On histamine in cotton dust and in the blood of cotton workers. J. Hyg. *37,* 234, 1937.

Lembeck, F. & Neuhold, K.: Nachweis von 5-Oxytryptamin im Harn. Arch. exp. Path. Pharm. *226,* 456, 1955.

Lowry, O. H., Graham, H. T., Harris, F. B., Priebat, M. K., Marks, A. R. & Bregman, R. U.: The chemical measurement of histamine in the blood plasma and cells. J. Pharmacol. Exper. Ther. *112,* 116, 1954.

McIntire, F. C., Roth, L. W. & Sproull, M.: In vitro histamine release from sensitized rabbit blood cells. Evidence against participation of fibrinolysin. Proc. Soc. Exper. Biol. Med. *73,* 605, 1950.

Perlman, E.: A quantitative method for the determination of antihistamine compounds containing the pyridineradical. J. Pharmacol. Exper. Ther. *95,* 465, 1949.

Pernow, B.: Studies on substance P. Purification, occurrence and biological action. Acta Physiol. Scandinav. *29,* Suppl. 105, 1953.

Pernow, B. & Waldenström, J.: Paroxysmal flushing and other symptoms caused by 5-hydroxytryptamine and histamine in patients with malignant tumours. Lancet *267,* 951, 1954.

Rhone — Poulenc, Specia 1954.

Rocha de Silva, M.: Histamine, its role in anaphylaxis and allergy. Thomas: American Lecture Series, 1955.

Rose, B. & Weil, P.: Blood histamine in the rabbit during anaphylactic shock. Proc. Soc. Exper. Biol. Med. *42,* 494, 1939.

Sjoerdsma, A., Weissbach, H. & Udenfriend, S.: A clinical, physiologic and biochemical study

of patients with malignant carcinoid (Argentaffinoma). Am. J. Med. *20,* 520, 1956.

Udenfriend, S. 1955. Cit. Langemann, H. & Kägi, J.: Oxythyptamin- und Oxyindolessigsäurebestimmungen bei einen Fall von Carcinoidsyndrom, nebst einigen anderen Untersuchungen über Oxytryptamin. Klin. Wchnschr. *34,* 237, 1956.

Valentine, W. N. & Lawrence, J. S.: Studies o blood histamine; partition of blood histamir before and after clotting in health and diseas states. Am. J. Med. Sci. *216,* 619, 1948.

Werle, E., Kehl, R. & Koebke, K.: Ueber Brady kinin, Kallidin und Hypertensin. Biochem. Z *320,* 373, 1950.

ADDENDUM

Recently Adam, Hardwick & Spencer (1957) have used Amberlite IRC-50 and XE-64 for extraction of histamine in human blood plasma. The blood, obtained from an antecubital vein, was allowed to run freely into a chilled polyethylene bottle, containing 0.9 per cent NaCl and heparin. The plasma was separated by centrifugation at + 4° C. By this technique the histamine equivalen of human plasma was found to be less than 1 n per ml.

Adam, H. M., Hardwick, D. C. & Spence K. E. V.: A method of estimating histamine i plasma. Brit. J. Pharmacol. *12,* 397, 1957.

dinav. J. Clin. & Lab. Investigation 241—245, 10, 1958.

DETERMINATION OF BILIRUBIN IN SERUM AS ALKALINE "AZOBILIRUBIN"*

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n the course of time many modifications Hymans van den Bergh's method for ntitative determination of bilirubin in um have been proposed and new ones are lished every day. Nearly all of these use red color of the neutral solution for rimetry or photometry and it seems as he excellent method described by Jend-sik & Gróf in 1938 advocating reading a strongly alkaline solution has been for-ten. This method was originally designed the Zeiss Pulfrich photometer, but may ily be modified for the spectrophotometer described below. The author has used this hod almost daily for several years and it be characterized as simple, rapid and able. All results obtained with the method easily reproduced. This view is held also With (1954) in his monograph where he cribes it as being "both specific and ntitative".

hrlich (1883) observed that bilirubin coupled diazotised sulphanilic acid and that the pigment ned was red in neutral and blue in alkaline and solutions. Hymans van den Bergh & Snapper 3) used this "azobilirubin" for quantitative rmination of bilirubin in human serum. Hymans van den Bergh & Müller (1916) discovered the accelerator effect of alcohol with certain sera and Adler & Strauss (1922) found that not only alcohol but also caffeine-sodium-benzoate and other compounds could serve as accelerators.

Jendrassik & Gróf (1938) used diazotised sulphanilic acid which gives water soluble pigments and the caffein-sodium-benzoate accelerator whereby they excluded precipitation of proteins by alcohol. They prefered to read in alkaline solution to avoid protein precipitation and because the specificity of the test is greatly increased when the red color is changed into a blue one. The color of the alkaline azobilirubin is often described as green, but this is because the blue pigment is mixed up with yellow pigments, formed when diazotised sulphanilic acid in alkaline solution couples with itself or caffein. The alkaline pigment in serum has its maximum absorption at 600 mμ where the absorption of the yellow non-bilirubin pigments of serum and several red and brown pigments preformed or formed by the reagents is negligible.

METHODS

Reagents

Reagent I: Caffein 20 gm, sodium benzoate 30 gm, sodium acetate 50 gm, distilled water ad 400 ml.

Reagent II a: Sulphanilic acid 0.5 gm, conc. hydrochloric acid 1.5 ml, distilled water ad 100 ml.

This investigation has been supported by grants n the Norwegian Research Council for Science the Humanities.

Reagent II b: Sodium nitrite 0.5 per cent solution in distilled water (not more than 14 days old).

Reagent II: 5 ml reagent II a and 3 drops of reagent II b (freshly prepared).

Reagent III: Sodium hydroxide 30 gm, sodium potassium tartrate 105 gm, distilled water 300 ml.

Technique

The tubes A, B and C are employed as seen from the table below:

Tubes	A	B	C
Distilled water	0	0.5	1 ml
Serum	1	1	0 ml
Reagent I	2	2	2 ml
Reagent II	0.5	0	0.5 ml

The contents are mixed and allowed to stand for 10 minutes at room temperature. Then 1.5 ml of reagent III is added to each tube. The extinctions (E_A, E_B, E_C) at 600 mμ were then measured in a Beckman B photoelectric spectrophotometer in 10 mm thickness.

COMMENTS

The bilirubin concentration in mg per 100 ml serum may now be calculated from the equation:

$$C = 4.50\ (E_A - E_B - E_C)$$

Tube B is a blank without the diazoreagent (the same blank extinctions are obtained with distilled water and diluted hydrochloric acid) and tube C is a blank which eliminates the yellow color formed by the diazoreagent and the caffeine in alkaline solution. E_C is, however, very small and may be neglected if E_A exceeds 0.300 (with icteric sera when undiluted).

Fig. 1 shows that $(E_A - E_B)$ has its maximum at 600 mμ and that E_C is very small at this wavelength, and Fig. 2 that Beers law is valid. Reagents I and II and the nitrite content of reagent II may be varied to a

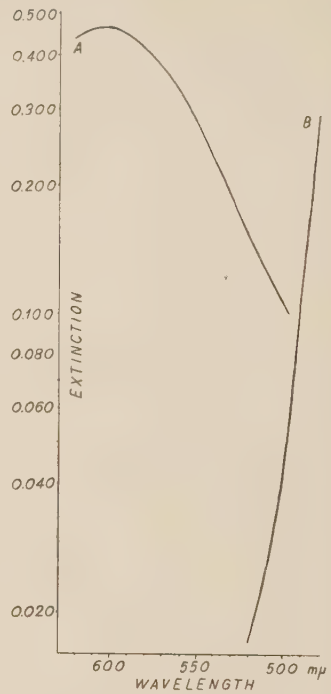

Fig. 1. Absorption curves. Alkaline "azobilirubin" in serum (A) and the reagent mixture (B). Bilirubin was dissolved in Na OH, serum was added and the mixture neutralized with hydrochloric acid. The analyses were performed with a blank treated in the same way but without bilirubin. The blank (B) was read against water.

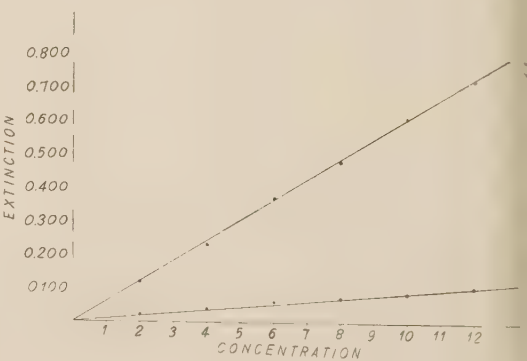

Fig. 2. Two dilution series which show that Beer's law is valid. Bilirubin was dissolved in serum as described in Fig. 1, and diluted with physiologic saline.

ble I. *Experiments with different ic- sera and bilirubin dissolved in normal n. The amount of reagents is varied e 0.5 ml serum is used instead of 1 ml the final volume made up to 5 ml with iologic saline. Only the reagent men- ed at the head of the columns is varied hile the other reagents are as given in the text.*

Reagents						
I		II		II b	III	
E_{600}	ml	E_{600}	dr.	E_{600}	ml	E_{600}
					1.1	0.244
					1.2	0.243
0.269	0.3	0.177	1	0.358	1.3	0.246
0.268	0.4	0.177	2	0.362	1.4	0.247
0.268	0.5	0.179	3	0.356	1.5	0.243
0.273	0.6	0.179	4	0.363	1.6	0.250*
0.268			5	0.359	1.7	0.254*

Turbid.

ble II. *The effect of the elapse of before addition of reagent which stops the reaction.*

Min.	E_{600}
2	0.445
4	0.447
6	0.439
8	0.438
10	0.438
15	0.441
20	0.442

t extent without affecting the result if final volume is made up to 5 ml, e reagent III gives turbidity in excess ble I). The limit of 10 minutes may be ed within several minutes (Table II), the reading has to be performed within minutes to avoid turbidity (Table III).

Table III. *The effect of the elapse of time after the addition of reagent III.*

Min.	E_{600}
1	0.312
10	0.314
20	0.312
30	0.310
40	0.315*
50	0.320*
60	0.330*

* Turbid.

Table IV. *The effect of different amounts of serum proteins. A solution of bilirubin in serum (dissolved as described in Fig. 1) was diluted with normal serum and physiologic saline (serum dilution 1/50 indicates that the one ml used in the reaction contains only 1/50 ml serum). Extinctions have been corrected for the amount of bilirubin present in the normal serum.*

Serum dilution	E_{600}	Serum dilution	E_{600}
1/1.1	0.363	1/5.0	0.368
1/1.2	0.372	1/5.6	0.365
1/1.4	0.393	1/6.3	0.376
1/1.6	0.367	1/7.1	0.369
1/1.9	0.373	1/8.3	0.373
1/2.4	0.368	1/10	0.371
1/3.1	0.374	1/12.5	0.365
1/4.5	0.367	1/17	0.366
1/8.3	0.365	1/25	0.365
		1/50	0.364
Sum			7.024
Mean			0.3697
Standard deviation			0.0068
Coefficient of variation			2 %

The method was tested with various dilutions of serum, too, and Table IV gives extinctions read with serum dilutions from 1 to 50 when the one ml used in the analysis contained a constant amount of bilirubin. The standard deviation of these 19 analyses was about 2 per cent of the mean. In a test

Table V. *Variation in triple analyses (a, b, c) with five sets of pipettes (1—5). Bilirubin dissolved in serum as described in Fig. 1.*

a	b	c
0.187	0.188	0.185
0.189	0.189	0.188
0.187	0.187	0.187
0.193	0.191	0.194
0.193	0.190	0.192
Sum		2.840
Mean		0.1893
Standard deviation		0.0027
Coefficient of variation		1.4 %

with five sets of pipettes and three analyses with each set (a total of 15) the coefficient of variation was 1.4 per cent. Forty-three double analyses had a mean difference smaller than 1 per cent.

The extinction quotient of pure bilirubin in serum was determined by dissolving bilirubin (Hoffmann La Roche) in chloroform, reading the extinction at 450 mμ with chloroform as blank, evacuating rapidly in vacuum, dissolving the residue in 0.1 normal solution of sodium hydroxide, adding normal serum poor in bilirubin, neutralizating with 0.1 N hydrochloric acid and then performing the analyses as described above with a blank treated in the same way, but without bilirubin. The millimolecular extinction quotient of bilirubin in chloroform has been determined by a number of authors and their values vary only from 55 to 56 (Lemberg & Legge, 1948). Bilirubin has the molecular weight 584.6 and the concentration in mg per 100 ml of bilirubin is then given by the equation:

$$C = \frac{584.6 \cdot E_{450}^{CHCl_3}}{55.5 \cdot 10} = k \cdot E_{600}^{azo}$$

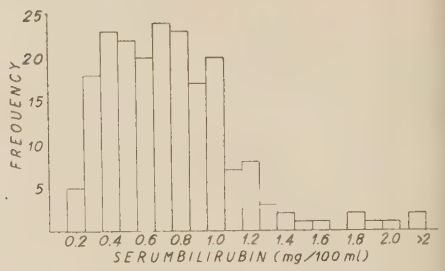

Fig. 3. Serum bilirubin in normal persons. 2(medical patients without injury to the liver or bi passages or hemolytic anemia. No sex or age di ference was observed (redrawn from the valu published by With, 1943).

With three different sera, k was found to b 4.49 — 4.37 — 4.43 — 4.45 — 4.54 — 4.4 — 4.40 — 4.73 — 4.63 with 4.50 as a mea An icteric serum diluted with physiolog saline (10 dilutions) was read both with tl Zeiss Pulfrich photometer and the spectr photometer. The mean was found to be 4.3 which agrees within the error of analys with the values given above.

With (1943) has published normal valu obtained with this technic employing t Zeiss Pulfrich photometer and Fig. 3 is re rawn with his values. They show a mea about 0.65 mg per 100 ml and the chara teristic asymmetry with higher values whi is probably due to cases of constitution hyperbilirubinemia.

SUMMARY

Jendrassik & Gróf's method is reviewe tested and modified for the spectrophot meter. The great advantages of this meth are emphasized and especially the great i crease in specificity obtained with the alk line pigment.

REFERENCES

r, E. & Strauss, L.: Beitrag zum Mechanismus der Bilirubinreaktion im Blut (II Mitteilung). Klin. Wchnschr. *1,* 2285, 1922.

ich, P.: Sulfodiazobenzol, ein Reagens auf Bilirubin. Centralbl. f. klin. Med. *4,* 721, 1883.

ıans v. d. Bergh, A. A. & Snapper, J.: Die Farbstoffe des Blutserums. Dtsch. Arch. f. klin. Med. *110,* 540, 1913.

& Müller, P.: Über eine direkte und eine indirekte Diazoreaktion auf Bilirubin. Biochem. Ztschr. *77,* 90, 1916.

Jendrassik, L. & Gróf, P.: Vereinfachte photometrische Methoden zur Bestimmung des Blutbilirubins. Biochem. Ztschr. *297,* 81, 1938.

Lemberg, R. & Legge, J. W.: Haematin compounds and bile pigments. Interscience Publ. Inc., New York, 1949, p. 122.

With, T. K.: Studies on serum bilirubin. Analytical methods and normal values. Acta Med. Scandinav. *115,* 542, 1943.

— Biology of bile pigments. Arne Frost-Hansen. Publ., Copenhagen, 1954, p. 154.

Scandinav. J. Clin. & Lab. Investigation 246—250, 10, 1958.

ICTERUS INDEX DETERMINED WITH THE SPECTROPHOTOMETER, CORRECTED FOR TURBIDITY AND HEMOGLOBIN*

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The spectrophotometric determination of the yellow color of serum (icterus index) may be relied upon only if adequate attention is paid not only to the presence of hemoglobin but also to turbidity. Most authors therefore have used serum extracts (alcohol, chloroform or acetone), but these give too low values because of the unknown amount of yellow pigment taken down with the protein precipitate.

Heilmeyer & Wappler (1928) were the first to determine the yellow color of whole serum. They found that the errors caused by hemoglobin and turbidity were less with the blue than with the red and green filters. Davies & Sheard (1934), Boggs (1954) and Shinowara (1954) corrected for hemoglobin only and Fog (1949) described a method with the Zeiss Pulfrich photometer correcting for turbidity. It must, however, be emphasized that the Shinowara-method, recently published and used by Svenneby (1956) does not make reservation for estimations in turbid sera. Shinowara finds too, that bilirubin in plasma has its maximum absorption at 450 mμ, while it is a well known fact confirmed by numerous authors (Lemberg & Legge, 1948; With, 1954) that bilirubin in serum has its maximum absorption at 460 mμ. The findings are explainable, however, because turbidity tends to give higher extinctions with the shorter wavelengths (Fog, 1952) and therefore presumably may change the maximum from 460 to 450 mμ. Standard solutions with maximum absorption at 450 mμ are not comparable with human sera.

The author (Fog, 1952) has published a method for correcting for turbidity in spectrophotometric analyses and with a precision within the usual limits of accuracy of spectrophotometric technic. This method was founded on the observation that the absorption curves of turbidities as they usually appear in biological samples very closely follow a straight line when plotted in logarithmic coordinates. The method for spectrophotometric determination of icterus index described here is based on this property and on the characteristics of the absorption curve of oxyhemoglobin.

Fig. 1 shows the absorption curves of oxyhemoglobin (B) and bilirubin in human serum (A) plotted with logarithmic coordinates. The oxyhemoglobin curve has its typical absorption maxima at 577 and 540 mμ and the very high maximum at about 414 mμ

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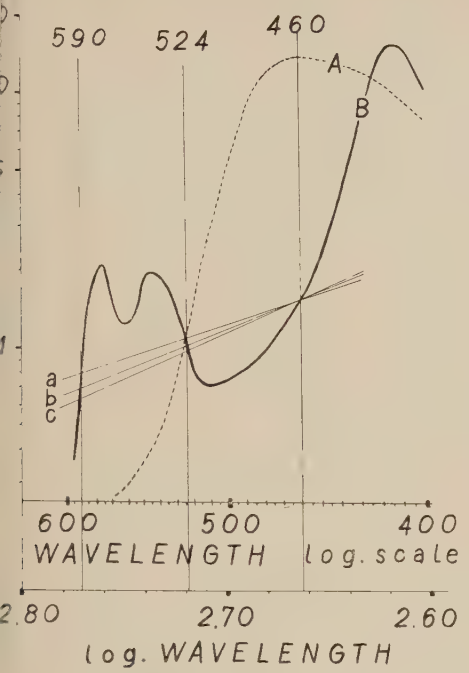

1. Absorption curves of oxyhemoglobin (B) bilirubin in serum (A). Both the ordinate and abscissa are drawn on logarithmic scales. Red d corpuscles washed with physiologic saline hemolysed by distilled water and read against r. Bilirubin (Hoffmann La Roche) was dis- d in 0.5 per cent sodium carbonate solution and al serum was added to convenient strength of yellow color and then read with the normal n, treated in the same way, but without bili- , as a blank. The lines a and c illustrates the s of E_{460}/E_{590} with different oxyhemoglobin ions and b the mean line. All extinctions in article were read in a Beckman B photoelectric rophotometer in 10 mm thickness.

raight line drawn between the extinctions at 590 and 460 mμ on the oxyhemo- in curve, crosses this curve at a third t at the wavelength 524 mμ. With this em of coordinates (which may be con- cted as a monogram, as described below) oxyhemoglobin absorption curve is independent of concentration, the socalled "typical absorption curve" and the crossing at 460 mμ of a line drawn through the extinctions at 590 and 524 mμ will give the oxyhemoglobin extinction at 460 mμ quite independent of the presence of a greater or smaller amount of hemoglobin in the solution. But extinctions read at 590 and 524 mμ may be very small, especially in diluted sera, and a nomogram (constructed as the three vertical lines in Fig. 1) then gives very incorrect values (see below under Theoretical considerations). A "calculation trick" is then introduced by adding an arbitary "theoretical turbidity" to diminish the nomogram error.

Example:

Read extinctions

$E_{590} = 0.082$ $E_{524} = 0.173$ $E_{460} = 0.843$

"Turbidity" added	0.100	0.139	0.200
Sum	0.182	0.312	1.043
Correction factor read from the nomogram E_{460}			0.560
			$E^{corr}_{460} = 0.483$

This procedure is used when E_{590} is less than 0.100. The most convenient "turbidity" for this purpose was found by examining 15 random sera which showed a ratio E_{524}/E_{590} varying between 1.10 and 1.66. With oxyhemoglobin in alkaline solutions the same ratio varied between 1.30 and 1.65. A ratio of 1.39 gives the following values from the nomogram:

$E_{590} = 0.100$ $E_{524} = 0.139$ $E_{460} = 0.200$, used in the example given above.

TESTS ON THE ACCURACY

Bilirubin was dissolved in normal serum (see text to Fig. 1) and 6 dilution series with a total of 42 tests were read with the diluted

normal sera as blanks. The values obtained were treated as described in the example above and the ratios $E_{460}/E_{460}^{corr.}$ were found to have a standard deviation between 1.9 and 3.2 per cent of the mean (coefficient of variation). In two other series (a total of 44 analyses) icteric serum was diluted with physiologic saline. The ratio conc./$E_{460}^{corr.}$ gave coefficients of variation 1.9 and 3.1 per cent.

It is shown in Table I that none of the analyses deviates more than 4 per cent from the mean when different quantities of oxyhemoglobin are added to normal turbid serum. Table II shows this experiment repeated with an icteric serum which gives a maximum deviation from the mean about ± 1 per cent. Table III shows an icteric serum diluted with physiologic saline (a) and distilled water (b) whereby the solution will be turbid because of the salting-in-effect precipitating certain proteins. None of the sets of values in the two series differs more than 3.5 per cent from their mean.

Table I. *Different amounts of oxyhemoglobin added to a constant volume of normal serum and made up to the same volume in all tests. The oxyhemoglobin solution was prepared as described in the text to Fig. 1.*

E_{590}	E_{524}	E_{460}	E_{460}	$E_{460}^{corr.}$
0.363	0.467	0.872	0.610	0.262
0.391	0.489	0.898	0.630	0.268
0.379	0.478	0.888	0.615	0.273
0.366	0.473	0.889	0.625	0.264
0.398	0.502	0.921	0.655	0.266
0.375	0.485	0.913	0.645	0.268
0.385	0.478	0.882	0.605	0.277
0.392	0.485	0.888	0.610	0.278
0.401	0.492	0.901	0.620	0.281
0.404	0.504	0.905	0.645	0.260
0.418	0.522	0.933	0.670	0.263
0.421	0.539	0.973	0.705	0.268
Mean				0.269
Standard deviation				0.0068
Coefficient of variation				2.5 %

Table II. *Different amounts of oxyhemoglobin added to a constant amount of icteric serum and made up to the same volume. The oxyhemoglobin solution was prepared as described in the text to Fig. 1.*

E_{590}	E_{524}	E_{460}	$E_{460}^{corr.}$
0.022	0.073	0.985	0.795
0.040	0.098	1.022	0.802
0.049	0.112	1.043	0.798
0.060	0.129	1.061	0.786
0.076	0.152	1.096	0.791
0.086	0.169	1.120	0.785
Mean			0.793
Standard deviation			0.006
Coefficient of variation			0.9 %

Table III. *Icteric serum diluted with distilled water (a) and physiologic saline (b). The salting in effect gives turbidity when serum is diluted with distilled water*

	E_{590}	E_{524}	E_{460}	E_{460}^{cor}
a	0.182	0.251	0.684	0.32
b	0.046	0.084	0.478	0.31
a	0.194	0.261	0.623	0.26
b	0.038	0.069	0.386	0.26
a	0.184	0.246	0.536	0.20
b	0.030	0.052	0.296	0.20
a	0.126	0.171	0.375	0.13
b	0.020	0.036	0.198	0.13
a	0.073	0.100	0.213	0.07
b	0.012	0.019	0.104	0.07

THEORETICAL CONSIDERATIONS

If the extinction (E) and the wavelength (λ) both are plotted with logarithmic coordinates, a straight line corresponds to the equation: $\log E = \log k + y \log \lambda$ or $E = k \lambda^y$ where k and y are constants indicating respectively the position and the slope of the line.

Serum containing both bilirubin (extinction E^B), hemoglobin (E^H) and showing turbidity (E^T) will show the following extinctions E_1, E_2 and E_3 at the wavelengths 590, 524 and 460 mμ respectively. Then

$$E_1 = E_1^B + E_1^H + E_1^T$$

$$E_2 = E_2^B + E_2^H + E_2^T$$

$$E_3 = E_3^B + E_3^H + E_3^T$$

Table IV. *Numbers obtained when an arbitary turbidity was added to extinctions obtained with bilirubin dissolved in serum to show the error introduced with the correction.*

Bilirubin in serum			Bilirubin in serum + turbidity					
E_{590}	E_{524}	E_{460}	E_{590}	E_{524}	E_{460}	ΔE_{460}	$E_{460}^{corr.}$	$E_{460}^{corr.}/E_{460}$
.022	0.150	2.000	0.122	0.289	2.200	0.730	1.470	0.735
.016	0.112	1.500	0.116	0.251	1.700	0.585	1.115	0.743
.011	0.075	1.000	0.111	0.214	1.200	0.440	0.760	0.760
.008	0.056	0.750	0.108	0.195	0.950	0.375	0.575	0.765
.005	0.037	0.500	0.105	0.176	0.700	0.310	0.390	0.780
.004	0.025	0.333	0.104	0.164	0.533	0.273	0.260	0.780
.002	0.015	0.200	0.102	0.154	0.400	0.245	0.155	0.775
.001	0.008	0.100	0.101	0.147	0.300	0.222	0.078	0.780

ecause the slope of the hemoglobin correction s chosen to approximate that of mean turbidity:

$$E_1^B + E_1^{HT}$$
$$E_2^B + E_2^{HT}$$
$$E_3^B + E_3^{HT}$$

re E^{HT} values are found on a straight line with coordinates given in Fig. 1 with a very small r which further decreases with a low hemo- in content of serum.

Vith bilirubin as the third factor the theory mes very complicated because a straight line vn through extinctions at 590 and 524 mμ ined with serum bilirubin has a slope very dif- it from the slope chosen for hemoglobin.

ut as a matter of fact the errors are found e astonishing small as shown by the figures n above and the numbers below calculated on tion of theoretical "turbidity" extinctions to ictions of bilirubin obtained as the mean of 40 with bilirubin dissolved in serum (cf. Fig. 1). n serum bilirubin $E_{460}/E_{524} = 13.3$ and $E_{460}/$ = 94.4. There is an overcorrection with higher es which increases to about 6 per cent with = 2.000 while extinctions below 0.500 give t values. If the turbidity values added are led $E_{460}^{corr.}$ will be correct to about $E_{460} = 1.000$ accuracy then decreases with lower values.

CONSTRUCTION OF NOMOGRAM

he best way to construct the nomogram is by use of a semilogarithmic paper with a decade on each 9—10 cm as ordinate and a cm scale as abscissa (cf. Fig. 1). The following values are traced out on the abscissa 2.78—2.77—2.76 . . . 2.66 with one cm between each value. Lines are drawn parallel to the ordinate at log 590 = 2.7709 log 524 = 2.7193 and log 460 = 2.6628. A line drawn between the values for E_{590} and E_{524} crosses the 460-line to give the correction factor (ΔE_{460}) which has to be subtracted from the E_{460} read in the photometer ($E_{460}^{corr.} = E_{460} - \Delta E_{460}$).

SUMMARY

A method is described which makes it possible to correct both for turbidity and for hemoglobin when the maximum absorption of bilirubin in serum at E_{460} is used as a measure of icterus index. The accuracy of the method is tested to be 1.9—3.2 per cent expressed as coefficient of variation.

REFERENCES

Boggs, T. R.: Early and reliable guide in the prognoses and management of hemolytic disease of the newborn. Am. J. Dis. Child. *88*, 506, 1954.

Davies, G. E. & Sheard, C.: The spectrometric investigation and determination of bilirubin. J. Lab. & Clin. Med. *19*, 593, 1934.

Fog, J.: Icterus index determined with the Zeiss Pulfrich photometer in turbid serum and plasma. Scandinav. J. Clin. & Lab. Investigation *1*, 255, 1949.

Fog, J.: Turbidity in photometry. Correction for turbidity in photometric methods. Analyst *77*, 454, 1952.

Heilmeyer, L. & Wappler, B.: Klinische Farbmessungen. VI Mitteilung. Die normale Serumfarbe. Ztschr. f. d. Ges. Med. 63, 630, 1928.

Lemberg, R. & Legge, J. W.: Hematin compounds and bile pigments. Interscience Publ. New York, 1949.

Shinowara, G. I.: Spectrophotometric studies o blood serum and plasma. The physical deter mination of hemoglobin and bilirubin. Am. J Clin. Path. *24*, 696, 1954.

Svenneby, G.: Spektrofotometrisk bestemmelse a totalbilirubin i serum. Nord. Med. *55*, 765, 1956

With, T. K.: Biology of bile pigments. A. Fros Hansen Publ. Copenhagen 1954.

ndinav. J. Clin. & Lab. Investigation 251—256, 10, 1958.

ERUM BILIRUBIN AND THE YELLOW COLOR OF SERUM*

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It is generally accepted that the yellow lor of normal serum is caused by a number different pigments (Lemberg & Legge, 49, With, 1954) while the color of icteric rum mainly is due to bilirubin. But also r icteric sera there exists a discrepancy be- een the yellow color and the serum bili- bin as determined by coupling with dia- tised sulphanilic acid (Heilmeyer & Krebs, 30; Müller & Engel, 1930; Heilmeyer & oop, 1932; Verzar, Süllmann & Vischer, 34; With, 1945; Lups & Meyer, 1946; ups & Francke, 1947). Curiously enough inowara (1954) and others have missed is disagreement between the serum color d the bilirubin concentration.

The sources of error in determination of e serum color (icterus index) are turbidity lemonstrable as Tyndall-effect) and the resence of small amounts of hemoglobin ways present in serum, both giving too high lues. Several authors have tried to elimin- e these error by means of extraction (alco- l, chloroform or acetone) but unfortunately is gives too low values because unknown nounts of yellow pigment are taken down th the protein precipitate. Davies & Sheard (1934) and Shinowara (1954) have published methods eliminating the hemoglobin error, but neglecting the presence of turbidity and Fog (1949) a method which minimizes the turbidity error. Further, Fog (1958, a) showed that it is possible to determine the icterus index corrected for both hemoglobin and turbidity by means of a spectrophotometer. This is the only method taking both sources of error into account and it is used in the present investigation.

Serum bilirubin may be found too low if the coupling between bilirubin and diazotized sulphanilic acid is incomplete (lack of accelerator) or if protein is precipitated taking down some of the azopigment. These difficulties are overcome by the technic of Jendrassik & Gróf (1938) lately reinvestigated by Fog (1958, b). In this method the very strong accelerator caffein-sodium-benzoate excludes lack of coupling, the protein precipitate is avoided and the red compound is changed into a blue pigment by means of a strongly alkaline buffer. In this way the specificity of the method is greatly increased.

EXPERIMENTAL

Bilirubin (Hoffmann La Roche) was dissolved in a 5 per cent sodium carbonate solution and normal serum was added in excess to change the maximum absorption from 420 to 460 mμ, the

* This investigation has been supported by grants m the Norwegian Research Council for Science d the Humanities.

Table I. *The columns show the groups, number of analyses, the means of* $E^{corr.}_{460}$ *and* E^{azo}_{600}, *the regression coefficients (b) and the correlation coefficients (r) expressing the correlation between* $E^{corr.}_{460}$ *and* E^{azo}_{600}.

	Groups	Number of Tests	Mean $E^{corr.}_{460}$	Mean E^{azo}_{600}	b	r
I	Bilirubin dissolved in serum. Total	115	0.706	0.288	0.4654	0.984
I A	I diluted with serum	17	0.851	0.375	0.4827	0.977
I B	I diluted with physiologic saline	23	0.568	0.235	0.4960	0.986
I C	I with serumbilirubin below 1.05 mg per 100 ml	55	0.401	0.148	0.3619	0.973
II	Non-jaundiced patients	338	0.365	0.105	0.3153	0.830
III	Sera from jaundiced patients. Total	185	0.856	0.266	0.3162	0.882
III A	III undiluted sera	104	0.873	0.271	0.3039	0.789
III B	III diluted with physiologic saline (icteric sera)	81	0.836	0.260	0.3201	0.906

maximum at 460 mμ being typical for bilirubin dissolved in human serum (Lemberg & Legge, 1949; With, 1954).

To the bilirubin serum solution an amount of hydrochloric acid equivalent to the sodium carbonate was added and the carbon dioxide evacuated by the water-jet-pump. A blank was treated in the same way, omitting the bilirubin. The samples were diluted either with normal serum or with physiologic saline and were read against their blanks both in determining the icterus index (Fog 1958, a) and the bilirubin (Jendrassik & Gróf, 1938, modified by Fog, 1958, b). The diazoreagent was added both to the test and to the blank omitting a reagent blank.

Each test (115 altogether from 32 sets of stock solutions) then gave one extinction for the icterus index ($E^{corr.}_{460}$) and one for the serum bilirubin (E^{600}_{azo}) (group I). Tests with serum dilution (group I A and III A) and tests with dilution with physiologic saline (group I B & III B) were compared, *i. e.* a covariance analysis was carried out, and no significant difference was found either between means or regression coefficients (Table II). The whole of group I was taken as a sample of "normal values" (serum containing bilirubin only). Fifty-five of these tests showed values below the limit of normal serum bilirubin, 1.05 mg bilirubin per 100 ml, *i. e.* corresponding to an extinction E^{azo}_{600} at about 0.235 with 10 mm cell, and were called group I C. It should be noted that the correlation lines between $E^{corr.}_{460}$ and E^{azo}_{600} actually are slightly curved, but in the present investigation they are assumed to be straight lines.

Samples of blood were drawn from the patients before breakfast by puncturing the cubital vein as in the general routine of the hospital. The samples were stored at room temperature for 3—6 hours

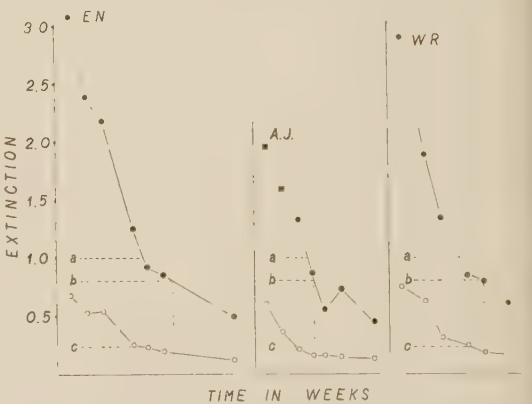

Fig. 1. Three cases of acute hepatitis (E. N., A. J., W. R.) with decreasing serumcolor. Serumcolor is indicated by ●●● as the extinction $E^{corr.}_{460}$ and serumbilirubin by ooo as the extinction E^{azo}_{600}. A serumcolor of about 10 units Meulengracht is indicated by a, and 8 units by b. The limit of normal serumbilirubin (1.05 mg per 100 ml) is indicated by c.

a b l e II. *The correlated groups are given the first column, the evidence of their ning from different populations in the last umn where ad. measures the statistical ociation between the adjusted means and measures the statistical association be- een the regression coefficients. The evi- nce of the groups coming from different pulations as great as or greater than one 1000 is indicated by ***, one in 100 by **, e in 100 by * and two in 10 no indicator.*

	Source of variation	Degrees of freedom	Mean square	Variance ratio
A	a	1	27	ad. 1624/27 = 60
	b	36	1661	
B	c	1	292	bc. 1661/292 = 5.7
	d	37	1624	
I A	a	1	105	ad. 5671/105 = 54
	b	181	5693	
I B	c	1	1594	bc. 5693/1594 = 3.7
	d	182	5671	
C	a	1	46990	ad. 46990/678 = 69***
	b	389	677	
	c	1	1693	bc. 1693/677 = 2.5
	d	390	678	
	a	1	401727	ad. 401727/4606 = 87***
	b	296	3839	
I	c	1	232111	bc. 232111/3839 = 60***
	d	297	4606	
	a	1	270130	ad. 270130/4628 = 58***
	b	192	3601	
I B	c	1	201862	bc. 201862/3601 = 56***
	d	193	4628	

fore the serum was separated. The tests were ranged in two groups. The first (group II) in- ded all who had not showed icterus during ir stay at hospital, while the second (group III) cluded all who had exhibited bilirubin values ove 1.05 mg per 100 ml once during their hospi- ization. Because of the high serum bilirubin in oup III, a great number of analyses were per- formed with serum diluted one to ten with physiologic saline. To ensure that no significant difference existed between undiluted (III A) and diluted (III B) sera, they were subjected to analyses of covariance (Table II) and no difference could be detected either between the means or the regression coefficients. They were consequently handled as a single group (III). The reason why all patients who had once during hospitalization suffered from hyperbilirubinemia were grouped together, is seen from Fig. 1 which is in agreement with the results obtained by several other authors (Kerppola, 1936; With, 1945; Lups & Francke, 1947) and shows that serum bilirubin, when decreasing, may reach the normal level even weeks before the icterus index.

The visible representation of the measurements given in Figs. 2 and 3 indicates, as confirmed by the analyses of covariance (Table II), that a difference exists between the means obtained from groups II and III relative to that of groups I C and I respectively.

This divergence must be due either to the presence of pigments which do not (or only incompletely) react with diazotized sulphanilic acid or to technical errors. As the "normals" and the tests were treated identically, it is unlikely that the correction technic should be responsible for the discrepancy between serum color and serum bilirubin. That the azobilirubin extinctions should be too low relative to the normals is also unreasonable as the same technic has been used with both groups. Only the presence of a substance retarding the coupling solely in icteric serum could explain such a discrepancy, but there is today no evidence for such a substance.

Dilution errors are out of the question as determinations of serum color and serum bilirubin always were performed in the same diluted or undiluted sample. A possible source of error is, however, oxidation of the

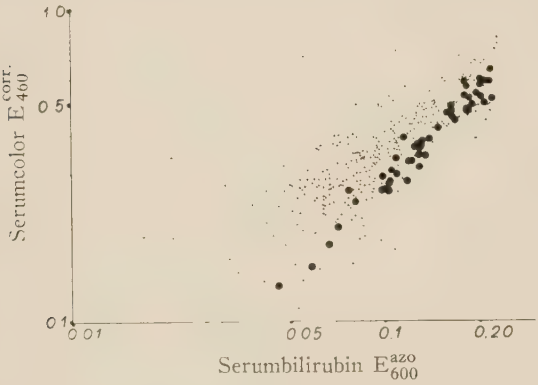

Fig. 2. Sera from patients who have not complained from icterus during hospitalization (+++) and bilirubin dissolved in normal sera (●●●). Ordinate $E_{460}^{corr.}$ and abscissa E_{600}^{azo} both with logarithmic scales.

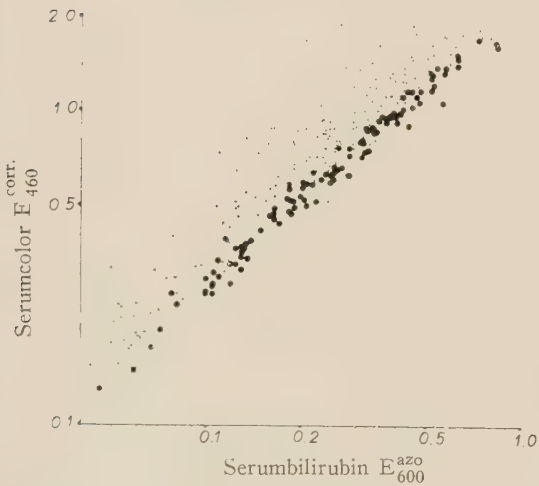

Fig. 3. Sera from patients who have been jaundiced during or just before hospitalization (+++) and bilirubin dissolved in normal sera (●●●). Ordinate $E_{460}^{corr.}$ and abscissa E_{600}^{azo} both with logarithmic scales.

bilirubin, as five to six hours have elapsed from the time the blood was drawn to the time when the analyses were performed. If some of the serum bilirubin is transformed to biliverdin during this period, the extinction at 460 mμ will show a slight decrease, but this will be followed by a decrease also in the coupling capacity with the diazoreagent as biliverdin does not couple. To exclude this possible error, extinctions were determined both at 620 mμ and 590 mμ and E_{620} never exceeded E_{590} as with biliverdin. Several sera were treated with sodium hypersulfite ($Na_2S_2O_4$) whereby biliverdin is reduced to bilirubin with an increase of E_{460}, but this extinction usually decreased, presumably because traces of oxyhemoglobin are reduced to hemoglobin. The presence of biliverdin in amounts which can explain the serum color bilirubin divergence is then unlikely.

Consequently there is a great gap between the intensity of yellow color and the bilirubin content both in normal and icteric sera.

DISCUSSION

That normal sera contain several yellow pigments in addition to bilirubin is quite reasonable, but in icteric sera the difference is so great that yellow pigments different from bilirubin (Hoffmann La Roche) must accumulate in the body in jaundice. To get an idea of the magnitude of these unknown factors $E_{460}^{corr.}$ for the yellow non-bilirubin was calculated according to the regression lines with $E_{600}^{azo} = 0.275$ which is the mean for groups I and III taken as a whole. The $E_{460}^{corr.}$ values are then calculated to 0.677 and 0.883 respectively with a difference of 0.206 — corresponding to the yellow non-bilirubin being equal to about 30 per cent of the total yellow color. With a serum of for example 200 (Meulengracht units) this makes up for about 60, which is really a very high serum color in itself. The difference between the regression coefficients obtained

h the normal and the icteric sera (group nd III) could be due to inhomogeniety of up III. But when group I is compared h group III B which contains only icteric a, the regression coefficients are still nificantly different. This also speaks inst a systematic error and indicates that yellow non-bilirubin pigments accumulate ependently of, but together with bilirubin l, as indicated in Fig. 1, "non-bilirubins" m to be eliminated more slowly than rubin.

There is no significant difference in nor- l sera concerning the regression coeffi- nts, indicating that the fluctuations in the rmal serum color probably are due to rubin, except of course in strict vege- ans who occasionally may accumulate otinoids even to the degree of pseudo- erus. Bilirubin seems to be the dominant ment even in normal sera comprising an erage of about 70 per cent of the serum ors.

This investigation shows that the results ained by With (1945) were correct and not be explained as caused by hemoglobin Müller, 1932; Lups & Francke, 1947) and also confirms the conclusions drawn by eilmeyer & Krebs (1930), Müller & gel (1930), Heilmeyer & Toop (1932), rzar *et al.* (1934), Lups & Meyer (1946) d Del Bono (1949).

The results obtained by Shinowara (1954), o used a spectrophotometer and found cellent correlation with icteric samples and poor correlation with non-icteric samples, e not confirmed. This is probably due to overcorrection with Shinowara's method cause turbidity will give a false and too gh correction factor for hemoglobin with this method. Svenneby (1956) confirmed Shinowara's results obtained with Shinowara's original technic. Peter (1933), Kerppola (1936), and Schwenkenbecher (1939) missed the discrepancy between serum color and the amount of bilirubin, but this is unquestionably due to inadequate technic.

SUMMARY

The yellow color of serum was determined with a spectrophotometer and a method was used which eliminates errors due to both hemoglobin and turbidity. The serum bilirubin was determined as alkaline azobilirubin. The correlation between serum color and azobilirubin was determined with normal sera (II), icteric sera (III) and bilirubin dissolved in normal sera (I). The two first groups were compared with the last group by means of covariance analysis. The fluctuations in the color of normal serum were found mainly to be due to varying amounts of bilirubin, which is the dominating yellow pigment of normal serum. Icteric sera contained greater amounts of yellow non-bilirubin pigments and these substances were shown to be eliminated more slowly than bilirubin.

REFERENCES

Bonnier, G. & Tedin, O.: Biologisk variationsanalys. Alb. Bonnier Publ., Stockholm 1940.

Bono, N. Del: Rapporti tra bilirubina serica e bilirubina cutanea negli itteri. Rev. di gastroenterol. *1,* 278, 1949. (Ref. With, 1954.)

Davies, G. E. & Sheard, C.: The spectrometric investigation and determination of bilirubin. J. Lab. & Clin. Med. *19,* 593, 1934.

Fog, J.: Icterusindex determined with the Zeiss Pulfrich photometer in turbid serum and plasma. Scandinav. J. Clin. & Lab. Investigation *1,* 255, 1949.

Fog, J.: Icterus index determined with the spectrophotometer corrected for turbidity and hemoglobin. Scandinav. J. Clin. & Lab. Investigation *10,* 246, 1958.

— Determination of bilirubin in serum as alkaline "azobilirubin". Scandinav. J. Clin. & Lab. Investigation *10,* 241, 1958.

Heilmeyer, L. & Krebs, W.: Spectrophotometrische Untersuchungen des Ehrlich-Pröscherschen Bilirubin-azofarbstoffes und ihre praktische Anwendung, besonders zur quantitativen Bestimmung des Bilirubins im Blutserum. Biochem. Ztschr. 223, 352, 1930.

Heilmeyer, L. & Toop, H.: Die spektrophotometrische Farbstoffanalyse des Blutserums mit besonderer Berücksichtigung des Bilirubin- und Carotin-spektrums. Ztschr. f. d. ges. Exper. Med., *80,* 603, 1932.

Jendrassik, L. & Gróf, P.: Vereinfachte photometrische Methode zur Bestimmung des Blutbilirubins. Biochem. Ztschr. *297,* 81, 1938.

Kerppola, W.: Ein kontrolliertes Verfahren zur quantitativen Bestimmung des Bilirubins. Die Diazofarbe und die Eigenfarbe des Serums als Masstab des Bilirubins. Acta Med. Scandinav. 89, 387, 1936.

Lemberg, R. & Legge, J. W.: Haematin compounds and bile pigments. Interscience Publ. Inc., New York, 1949.

Lups, S. & Meyer, F. G. D.: Some investigations about the occurrence of pigments that give no diazo reaction in jaundiced sera. Acta. Med. Scandinav. *126,* 85, 1946.

Lups, S. & Francke, C.: Observations on the occurence of yellow pigments different from bilirubin and carotinoids in sera of healthy persons and icteric patients. Acta Med. Scandinav. *129,* 234, 1947.

Müller, P. & Engel, L.: Über die Bestimmung des Gallenfarbstoffs im Blutserum. Klin. Wchnschr. *9,* 2304, 1930.

Müller, P.: Spektrochemische Analyse der Serumfarbstoffe. Klin. Wchnschr. *11,* 189, 1932.

Penati, F. & Pagliardi, E.: Ricerce spettrografiche sui pigmenti bilari del sangue humano in rapporto al al diverso tipo della reazione diazonica. Arch. sci. med. *76,* 21, 1943, ref. With 1954.

Peter, J. R.: Gesamtfarbstoff und Bilirubin im normalen Menschlichen Blutserum. Biochem. Ztschr. *262,* 432, 1933.

Schwenkenbecker, W.: Vergleichende stufenphotometrische Bilirubinbestimmungen im Serum bei verschiedenen Krankheiten. Ztschr. f. klin. Med. *136,* 116, 1939.

Shinowara, G. I.: Spectrophotometric studies on blood serum and plasma. The physical determination of hemoglobin and bilirubin. Am. J. Clin. Path. *24,* 696, 1954.

Verzár, F. & Süllmann, H. & Vischer, A.: Die Differenzierung der Farbstoffe des menschlichen Blutserums. Biochem. Ztschr. *274,* 7, 1934.

With, T. K.: On the occurrence in human serum of yellow substances different from bilirubin and carotinoids. Acta Med. Scandinav. *122,* 501, 1945.

— Occurrence in human serum of yellow substances different from bilirubin. Nature 158, 310, 1946.

— Biology of bile pigments. Arne Frost-Hansen Publ., Copenhagen, 1954.

ndinav. J. Clin. & Lab. Investigation 257—262, 10, 1958.

STUDIES OF THE CONGO RED BINDING TO SERUM*

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The use of the Congo red test for amydosis has called for a more thorough study the nature of the Congo red binding to serum proteins. The Bennhold Congo l test for amyloidosis (1923) measures proportion of this dye which is retained the plasma one hour after its intravenous ection. The validity of this test has often en the subject of intense discussion. Reitly this problem has been taken up in a per by Dixon, Ramcharan & Ropes (1955) o found that the retention of Congo red rheumatoid arthritis was decreased, incating a tendency of the Congo red test become positive in this disease. Another ngo red test for amyloidosis, the intrataneous Congo red test used to demonrate amyloid in the skin, as shown by ine (1956), also tends toward the positive rheumatoid arthritis. This test demonrates a prolonged persistence of intrataneously injected Congo red in the skin patients with rheumatoid arthritis.

In vitro studies of the affinity of serum r Congo red by Larsen (1958) showed that e potency of binding Congo red is lowered sera from patients with rheumatoid arthritis. Concurrently, determinations of the serum glycoproteins were carried out and an inverse proportionality between the Congo red binding to serum and the content of glycoproteins was noted.

The present investigation was undertaken in order to determine if it is reasonable to assume that the dye binding to serum is dependent on the presence of polysaccharide anions which could compete with the dye on the binding sites of the serum proteins. A competition of polysaccharide anions with anionic dyes for serum albumin is suggested by Klotz (1953), and Karuch (1951) has observed that several organic anions act competitively in the binding of dye anion to albumin.

Since Congo red does not penetrate semipermeable membranes, equilibrium dialysis cannot be used in the present study. The method devised in it is based on a partition analysis system. Cellulose powder, which has a great affinity for Congo red, was loaded with Congo red. Upon shaking this Congo red-loaded cellulose powder with a buffered serum solution, Congo red was taken up by the serum proteins.

* The investigation was supported by grants m The Danish League Against Rheumatism.

EXPERIMENTAL

Materials

The sera used in the present study are, 1) a normal serum pool consisting of 10 normal sera,[1] 2) a pool of rheumatic sera consisting of 37 sera from patients with manifest rheumatoid arthritis.[2]

Crystalline human serum albumin, gamma globulin and a globulin fraction containing: albumin +alpha$_1$-globulin 6.6 per cent, alpha$_2$-globulin 65.1 per cent, beta$_1$-+beta$_2$-globulin 18.1 per cent, and gamma-globulin 10.2 per cent[3] were used as 5 per cent solutions in m/100 phosphate buffer pH 7.4.

The Congo red, (Grübler & Co.) exhibited a molar extinction coefficient in phosphate buffer (m/100 pH 7.4) of $\varepsilon = 23100$ at λ max 495 mμ, and in buffer/serum (1 : 100) of $\varepsilon = 25550$ at λ max 505 mμ.

Toluidine blue (Riedel de Haen) exhibited $\varepsilon = 29600$ at λ max 635 mμ in phosphate buffer m/100 pH 7.4 measured at a concentration of $0.73 \cdot 10^{-5}$ M.

Competing agents. Sodium salicylate, sodium dodecylsulfate and the sodium salt of 3,5-dioxo-1,2-diphenyl-4n-butylpyrazoline, Butazolidin,[4] were used in final concentrations varying from $n=10^{-1}$ to $n=10^{-2}$. Sodium chloride was used in the same concentrations. Dextransulfate, sodium salt, (Dextran LTD. Aycliffe, Darlington, England) in final concentrations of 0.2 to 2.0 mg/ml and heparin, sodium salt,[5] were used in final concentrations of from 0.2 to 2.0 mg/ml.

Methods

Preparation of cellulose-Congo red suspension. 50 g cellulose powder (Schleicher & Schüll No. 123, for chromatography) is suspended in 200 ml of a 0.5 per cent Congo red solution in water. After stirring for two hours the suspension is filtered and washed with water until the filtrate is colorless. The washed powder is then suspended in $\frac{n}{20}$ phosphate pH 7.4.

Partition analysis. The Congo red is diluted 1 : 5 with water. From this diluted suspension 5 ml are taken with a Krogh syringe while stirring, and to these 5 ml are added 50 μl serum with a micropipette. 10—100 μl of the competing agents were added in buffered solutions, giving the final concentrations as mentioned above. The test tubes with Congo red suspension and serum are shaken together with a blank, for one hour at room temperature at a frequency of 150 cycles per minute. After centrifugation the supernatant is read against a blank in a Beckmann B. Spectrophotometer at 505 mμ, all in duplicate. The extinction of the blank, Congo red without serum added, should not exceed 0.030 extinction units. None of the competing agents exhibited extinction readings different from the blank.

The same method was used in determinations of the binding of toluidine blue to serum. The toluidine blue in the supernatant after shaking serum solutions with toluidine blue-loaded cellulose was read at 635 mμ within one hour. Standing at room temperature for longer periods results in a decoloration due to reduction of the dye by the serum.

RESULTS

In Fig. 1 is shown the result of plotting log. [serum] against log. ε at 505 mμ, which is the extinction of the Congo red taken up by the serum. Within the concentration range used in the experiments, the dye uptake is considered to obey the Freundlich adsorption law. It is noted that the normal serum has a greater binding affinity for Congo red than serum from patients with rheumatoid arthritis. When toluidine blue is used as shown in Fig. 2, where log. [serum] is plotted against log. ε at 635 mμ which is the extinction of toluidine blue, an increase in binding affinity to rheumatoid serum is noted, compared to normal serum.

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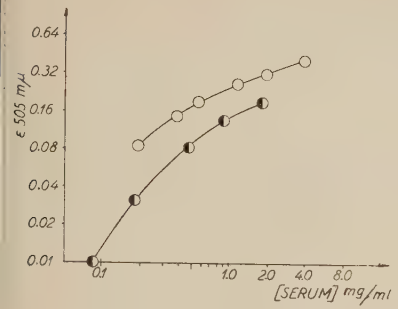
[1] Kindly furnished by the Blood Bank, University Hospital of Copenhagen.

[2] The author thanks Dr. J. Kryger, Copenhagen County Hospital, for collection of these sera.

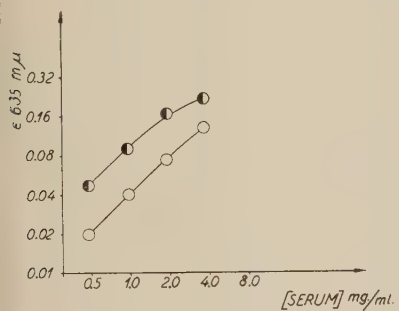
[3] Provided through the courtesy of State Serum Institute, Copenhagen.

[4] Kindly supplied by J. R. Geigy, AG. Basel.

[5] Kindly furnished by Leo Pharmaceutical Products, Copenhagen.



1. Congo red uptake of normal serum o—o serum from patients with rheumatoid arthritis —◐ Spectrophotometrically determined after ing serum dilutions in phosphate m/100, pH 7.4 with Congo red-loaded cellulose powder.



2. Uptake of toluidine blue by normal serum and by serum from patients with rheumatoid fitis ◐— ◐. Spectrophotometrically determined shaking serum dilutions in phosphate m/100, 7.4 with toluidine blue-loaded cellulose powder.

Addition of different organic anions to mal serum results in a decline in binding acity of Congo red to the serum, as shown ig. 3 and Fig. 4. Sodium chloride did not ibit any suppression of the Congo red ding, as shown in Fig. 3. A summary of effect of the organic anions on the Congo uptake by serum is shown in Table I, onstrating that dodecylsulfate is the ngest competing agent among the com- nds investigated in the present study. In centrations ten times that of dodecyl-

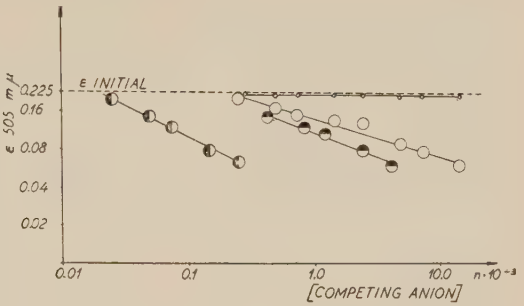


Fig. 3. Inhibition of Congo red uptake by normal serum due to presence of: Dodecylsulfate ◐—◐ Butazolidin ◓—◓ Salicylate o—o, and NaCl c—c.

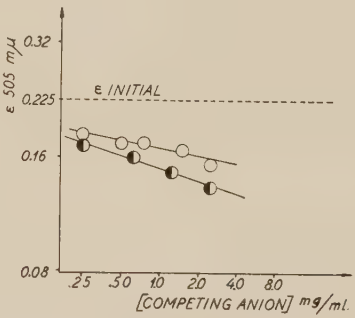


Fig. 4. Inhibition of Congo red uptake by normal serum due to presence of: Dextran sulfate ◐— ◐. and Heparin o—o.

Table I. *Concentrations of added anions* capable of lowering the Congo red binding to serum 10 per cent.*

Dodecylsulfate	$0.23 \cdot 10^{-3}$
Butazolidin	$2.4 \cdot 10^{-3}$
Salicylate	$2.7 \cdot 10^{-3}$
Dextransulfate	0.8 mg/ml
Heparin	1.3 mg/ml

* Sodium salts.

sulfate butazolidine and salicylate exhibit similar depressions of the binding potency of Congo red in the serum. The anionic concentrations of heparin and dextran sulfate were

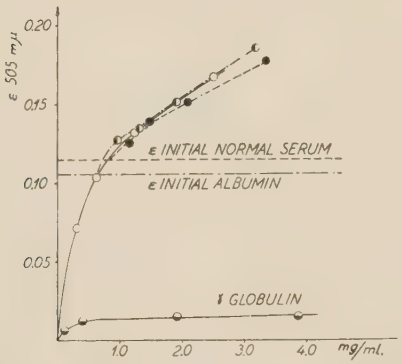


Fig. 5. Congo red uptake by a globulin fraction containing 65 per cent alpha$_2$-globulin o—o. Different amounts of the alpha$_2$-globulin fraction added to human serum albumin at a constant concentration of 0.313 mg/ml, ◐—◐. Alpha$_2$-globulin added to normal serum at a constant concentration of 0.438 mg/ml, ●—●. Congo red uptake by gamma globulin ◒—◒

not determined, and the concentration for significant competitive action of these compounds is therefore given in mg/ml.

An experiment was carried out to determine whether there might be interactions among the serum protein fractions. Crystalline human serum albumin and an alpha$_2$-globulin fraction both showed ability to take up Congo red, Fig. 5. In a concentration of 0.313 mg/ml, serum albumin exhibited the same Congo red uptake as did the alpha$_2$-globulin fraction at 0.690 mg/ml.

Gamma globulin did not bind Congo red to any considerable extent. Different amounts of the alpha$_2$-globulin fraction were added to albumin at a constant concentration of 0.313 mg/ml, and to normal serum at a constant concentration of 0.438 mg/ml. The dye uptake is shown in Fig. 5, where the initial points of the curves for the Congo red uptake by alpha$_2$-globulin+albumin and alpha$_2$-globulin+serum are transferred to the poi of the alpha$_2$-globulin curve having the sam ordinate. This makes comparison of the re sults easier. The curves show that the dy binding capacities for serum albumin an the alpha$_2$-globulin fraction are additiv whereas the sum of the dye-binding affinitie of serum and the alpha$_2$-globulin fraction greater than the binding capacity of a mix ture of these two components.

DISCUSSION

Complex formation between serum a bumin and organic anions has been intense studied by Klotz (1946), and Karuch (1951 Serum albumin has an outstanding ability form complexes with many compound among which the organic anions are mo reactive. The alpha$_2$-globulin fraction e hibited less affinity for Congo red than d albumin, but in the case of elevated alph globulin in the serum, this fraction may al be responsible for the Congo red uptak (Bennhold, Ott & Wieh, 1950). The obse vation that serum, to which the alpha globulin fraction is added, shows lower bin ing capacity for Congo red than expected the binding capacities of serum and t alpha$_2$-globulin were additive, suggests competition between anions from glyc proteins in the alpha$_2$-globulin and d anions. A similar competition could be a sumed to occur in the albumin+alpha globulin mixture, where a binding of pol saccharide anions to albumin might tak place. In the present experiments such an i hibition of Congo red uptake by albumin combination with the alpha$_2$-globulin fracti was not demonstrable.

he competing agents studied exhibited ferent abilities to block the cationic bind- sites on the serum proteins. 0.1 M sodium oride combines with serum albumin, as wn by Karuch (1950). In the present ly no competition was noted with sodium oride in concentrations up to 0.01 M. The at affinity of dodecylsulfate for serum umin was noted by Klotz (1946). Buta- din is found in the serum entirely as an on, as pointed out by Pulver & Herr- nn (1956), who also found that buta- din is demonstrable in the enol form in um. Wunderly (1956) showed that buta- din, although it is an organic anion, is nd to the alpha$_2$-globulin. Heparin and tran sulfate, as well as the low molecular anic anions, may combine with the serum teins. Naguchi (1956) has recently demon- ated that anionic polymers combine with tein to form soluble complexes even on alkaline side of the I.E.P., and has shown t serum albumin has roughly equal affini- for both anionic —COO^- and —SO^{-3} ups of polyelectrolytes.

n the pool of rheumatic sera a decreased ding of Congo red was noted. The lowe- serum albumin, which is characteristic of a from patients with rheumatoid arthritis, y influence the binding affinity of the a for Congo red (Rawson, 1943). Block- of the binding sites for Congo red to the um proteins by protein-bound lipids can, ording to Bennhold & Ott (1952), take ce in cases with increased lipids in alpha- beta globulin. In the present study is gested that increased concentration of ysaccharide anions may also block the ding sites for Congo red. This suggestion is supported by the demonstration of increased ability of the serum from patients with rheumatoid arthritis to bind toluidine blue, which indicates that rheumatoid serum contains more anionic binding sites than normal serum.

SUMMARY

The binding of Congo red and toluidine blue to normal serum and to serum from patients with manifest rheumatoid arthritis was studied by means of partition analysis by the uptake of dye from a suspension of dye-bound cellulose.

Serum from patients with rheumatoid arthritis showed decreased affinity for Congo red and increased affinity for toluidine blue compared to normal serum.

The binding of Congo red to normal serum is competitively blocked by the sodium salts of dodecylsulfate, salicylate, butazolidin, dextran sulfate and heparin.

An alpha$_2$-globulin fraction from human serum containing 65 per cent alpha$_2$-globulin exhibited a binding potency of about one-third that of albumin. In combination with albumin, the binding capacity of alpha$_2$-globulin is additive to the binding capacity of albumin. With serum the total binding capacity of serum+alpha$_2$-globulin was less than the sum of the binding capacities of the individual components.

On the basis of these observations it is suggested that the decreased affinity of serum for Congo red seen in serum with high glycoprotein content is partly due to an increase in total amounts of acid groups able to compete with the dye.

REFERENCES

Bennhold, H.: Über die Ausscheidung intravenös einverleibten Kongorotes bei den verschiedensten Erkrankungen insbesondere bei Amyloidosis. Dtsch. Arch. Klin. Med. *142,* 32, 1923.

— & Ott, H.: Die Globulinvehikel. Schweiz med. Wchnschr. *82,* 475, 1952.

— — & Wieh, M.: Über den Bindungunterschied lebergängiger und nierengängiger Substanzen an die Serumeiweiskörper. Dtsch. med. Wchnschr. *75,* 11, 1950.

Dixon, A. S. J., Ramcharan, S. & Ropes, M. W.: Rheumatoid arthritis: Dye retention studies and comparison of dye and radioactively labelled red cell methods for measurements of blood volume. Ann. rheum. Dis. *14,* 51, 1955.

Karush, F.: Heterogeneity of the binding sites of bovine albumin. J. Am. chem. Soc. *72,* 2705, 1950.

— The study of protein binding by partition analysis. The effect of protein charge. J. Am. chem. Soc. *73,* 1246, 1951.

Klotz, I. M.: Spectrophotometric investigations of the interaction of protein with organic anions. J. Am. chem. Soc. *68,* 2299, 1946.

Klotz, I. M.: In "The Proteins" ed. Neurath, H & Bailey, K. Vol. I. Acad. Press. 1953, p. 801

Laine, V.: An intracutaneous Congo red test i rheumatoid arthritis. Acta rheum. Scandinav. 2 31, 1956.

Larsen, B.: Relation between the affinity for cong red and the glycoprotein content of serum i rheumatoid arthritis and related diseases. An rheum. Dis. 17. June 1958.

Noguchi, Hajme: Interactions of proteins wit polymeric materials. Biochem. Biophys. Act *22,* 459, 1956.

Pulver, R., Exer, B. & Herrmann, B.: Über di Beeinflussung enzymatischer Reaktionen durc Phenylbutozon und die Übertragbarheit Fer mentchemischer Befunde auf die Stoffwechsel prozesse der Zelle. Schweiz. med. Wchnsch. *86* 1080, 1956.

Rawson, R. A.: Binding of T-1824 and structurall related diazo dyes by plasma proteins. Am. J Physiol. *138,* 108, 1943.

Wunderly, Ch.: Die Bindung von Butazolidin a die Serumproteinfractionen. Contemporar Rheumatology. Goslings and van Swaay, Am sterdam, 1956, p. 616.

dinav. J. Clin. & Lab. Investigation 263—266, 10, 1958.

DETERMINATION OF THE HEPATIC BLOOD FLOW BY MEANS OF ETHANOL PRELIMINARY REPORT

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(Received for publication January 25, 1958)

he blood flow through an organ may be rmined according to the Fick principle 70), if the concentration of a substance rring in the blood is altered by passing ugh this organ. The blood flow per min- may be calculated, from the quantity of substance metabolized per minute and difference in concentration between af- nt and efferent blood, as follows:

d flow per minute =

$$\frac{\text{quantity metabolized per minute}}{\text{change in concentration}}$$

ccording to this principle, methods for rmining the hepatic blood flow have n devised using bromsulphalein (Bradley, lefinger, Bradley & Curry, 1945), the en- enous urea synthesis (Myers, 1947), and ctose (Tybjerg Hansen, Tygstrup & nkler, 1954).

These methods, however, have required atic vein catheterization in order to obtain ples of efferent blood.

Numerous investigations appear to con- n the assumption that the oxidation of nol to acetaldehyde takes place largely, haps exclusively, in the liver (Elbel Schleyer, 1956). Consequently, ethanol uld be well-suited for determining the atic blood flow on the basis of the Fick principle, but no such experiments appear to have been published.

This paper describes attempts at determining the hepatic blood flow by means of ethanol.

METHOD

The experiments were carried out partly on patients, without demonstrable hepatic disease, admitted to the Surgical Department and partly on apparently healthy subjects (medical students).

The experiments were started in the morning with the subjects in the post-absorbtive state.

A solution of 60 g ethanol in 1000 ml isotonic sodium chloride was given intravenously.

During the entire procedure, the subjects were lying at rest. At regular intervals, 3 ml of blood was removed from an arm vein into a test tube containing 2 drops of heparin.

Ethanol was determined enzymatically on whole blood using alcohol dehydrogenase (Dotzhauer, Redetzki, Johannsmeier & Bücher, 1952). The standard deviation is 1.2 per cent.

Oxidation curves were drawn, plotting mg of ethanol in 100 ml of blood against time in minutes.

RESULTS

A typical oxidation curve is shown in Fig. 1.

After an initial rise, the descending limb of the curve may be divided into three parts.

The steep decline of the first part of the

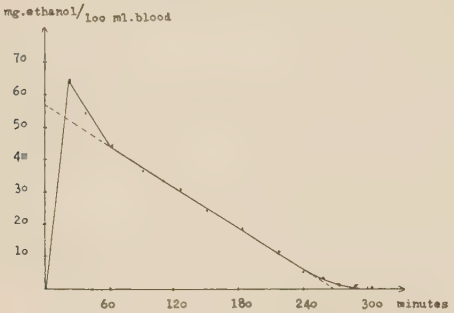

Fig. 1. Oxidation curve following i. v. infusion of 20.33 g ethanol in 10 min. $c_0 = 57.5$ mg. $c_{t1}—c_{t2} = 31.5—18.5$ mg ($t_1 = 120$ min. $t_2 = 180$ min.). Woman, aged 21, body weight 59 kg.

curve is explained by the oxidation of ethanol and its distribution in that part of the organism which is able to dissolve ethanol.

The second part shows the well-known rectilinear fall which is generally interpreted as evidence that the oxidation is constant per unit of time, independent of the concentration.

The third part begins 4 hours after the infusion has been discontinued and is characterized by a marked flattening of the curve, probably meaning that the liver is being supplied with less ethanol than it can oxidize.

In the calculations, no regard was paid to the relatively small quantities of ethanol excreted with the urine and respiratory air.

Extrapolation of the rectilinear part of the curve to the ordinate indicates the concentration (c_0), which would have been achieved if the amount administered had been distributed instantaneously in the body fluid.

The fall per minute in the concentration expressed in mg per 100 ml blood, may be read from the rectilinear part of the curve, expressed as:

$$\frac{c_{t1}—c_{t2}}{t_2—t_1}$$

The quantity of ethanol in mg oxidize per minute is obtained by multiplying th amount of ethanol administered with th quantity

$$\frac{c_{t1}—c_{t2}}{c_0 \times (t_2 — t_1)}$$

In the example given in Fig. 1 the quantit of ethanol oxidized is:

$$\frac{20330 \times (31.5—18.5)}{57.5 \times 60} = 77 \text{ mg/min.}$$

From the curve in Fig. 1 it may be cal culated that the deviation from the rectilinea course occurs at an ethanol level of 4.8 mg 100 ml blood, *i. e.* 48 mg ethanol per litre o blood. This is the minimum concentration a which a constant oxidation can be maintained

At a constant rate of oxidation, 77 m ethanol is oxidized per minute. If the live is to be supplied with this quantity, the bloo flow has to be 1.60 litre per minute (77 : 48)

Thus, the hepatic blood flow in litres pe minute may be calculated from an oxidatio curve as follows (I):

$$\frac{\text{oxidized ethanol in mg/min.}}{\text{mg ethanol/litre blood at the point of deviation from the rectilinear course.}}$$

The hepatic blood flow may, however, b determined more rapidly and, presumably more accurately, as follows:

A quantity of ethanol is infused in orde to obtain a blood level lower than that a which the curve flattens out. Ethanol is the infused at a rate which gives a quantity pe minute smaller than the amount oxidize per minute at blood levels corresponding t the rectilinear part of the oxidation curve.

If the rate of infusion is kept constan the curve will be horizontal, provided tha the hepatic blood flow is constant. Alteration in hepatic blood flow will appear as altera tions in the blood levels.

he concentration at which the curve be-
es horizontal is proportional to the quan-
of ethanol infused per minute and to the
tic blood flow. Accordingly, the blood
through the liver per minute may be
ulated as follows (II):

$$\frac{\text{inistered quantity of ethanol in mg/min.}}{\text{ethanol in blood in mg/litre}}$$

ig. 2 shows an example of this latter
nod of calculation (same subject as in
1).

fter infusion of 1.56 g ethanol in 5 min-
. 57 mg ethanol per minute is administe-
for 2 hours. At a concentration of 3.4
100 ml blood, the curve becomes hori-
al. The hepatic blood flow per minute
ng the experimental period is then 1.68
(57 : 34) (*cf.* 1.60 litre when calculated
rding to formula I).

DISCUSSION

xperiments which aim at confirming the
racy and applicability of these methods
in progress and will be published later.
he methods possess the immediate ad-
tage of not requiring hepatic vein cathe-
zation which involves a fairly great un-
ainty, the results depending to a great
nt on the position of the catheter tip in
liver. Besides, the catheterization makes
determination more cumbersome and may
e the patient inconvenience.
the present methods prove applicable,
technique is so simple that the determina-
of hepatic blood flow may be carried out
ny laboratory where a suitable photo-
er is available.

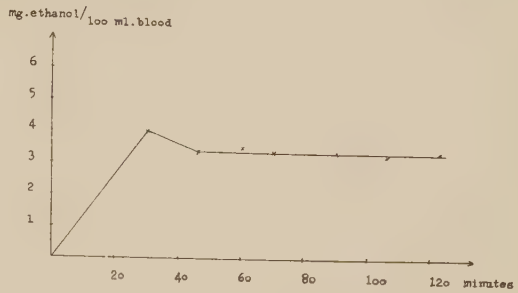

Fig. 2. Oxidation curve following continuous i. v. infusion of 57 mg ethanol per min. Initial infusion of 1.56 g ethanol in 5 min. Same subject as in Fig. 1.

It has previously been demonstrated that ethanol concentrations like those used in the present methods increase the hepatic blood flow (Mendeloff, 1954; Winkler, 1957). This need not, however, exclude the use of ethanol for determining the hepatic blood flow, as perhaps it affords a means of diagnosing impaired function.

Incidentally, ethanol in these determinations may be replaced by galactose, as the oxidation curves of these two substances display similar features (Tygstrup & Winkler, 1954). This procedure is now being tested.

SUMMARY

This is a preliminary report of two methods, not previously published, for determining the hepatic blood flow by means of ethanol, without using hepatic vein catheterization.

REFERENCES

Bradley, S. E., Inglefinger, F. J., Bradley, G. P. & Curry, J. J.: The estimation of hepatic blood flow in man. J. Clin. Investigation *24*, 890, 1945.

Dotzhauer, G., Redetzki, H., Johannsmeier, K. & Bücher, Th.: Erprobung einer spezifichen Fermentmethode zur Mikrobestimmung von Äthylalkohol. Deutsche Ztschr. ges. gerichtl. Med. 41, 15, 1952.

Fick, A.: Sitzungsb. Der Phys. Gesellsch. Z. Würtzb. 1870, p. 16.

Hansen, Tybjerg, A., Tygstrup, N. & Winkler, K.: Determination of the hepatic blood flow by galactose. Dan. Med. Bull. *1,* 146, 1954.

Mendeloff, A. J.: Effect of intravenous infusion of ethanol upon estimated hepatic blood flow in man. J. Clin. Investigation *33,* 1298, 1954.

Myers, J. D.: The hepatic blood flow and splanchnic oxygen consumption of man — Their estimation from urea production or bromsulphalein excretion during catheterization of the hepatic veins. J. Clin. Investigation *26,* 1130, 1947.

Tygstrup, N. & Winkler, K.: Kinetics of galactose elimination. Acta physiol. Scandinav. *32,* 354 1954.

Winkler, K.: Nyere undersøgelser om leverens blodforsyning. Månedsskrift for praktisk lægegerning og social medicin *3,* 112, 1957.

ndinav. J. Clin. & Lab. Investigation 267—271, 10, 1958.

A BUBBLE METHOD FOR DETERMINATION OF pCO_2 AND pO_2 IN BLOOD

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(Received for publication February 7, 1958)

Determination of the arterial pO_2 and O_2 are made by a modification of Krogh's bble method (1908). Principally, the de- rmination is carried out in two steps: The equilibration of an air bubble with e gases in the arterial blood, and 2) the alysis of the equilibrated bubble. Krogh rformed the first step in his microtono- eter in a flowing stream of blood. This pro- dure is, however, not possible when only limited amount of drawn arterial blood is ailable. After some trials the following thod which combines features of Krogh's thod with details from Riley's (1945) and mbertsen's (1952) was adopted:

Description of the method.

Arterial blood is transferred anaerobically to a nl all-glass syringe. The dead space of the syringe ntains a drop of mercury for stirring and the nger is slightly greased with paraffin oil to oid sticking. About 1 ml of blood is first drawn through a transfer tube and expelled again with from the transfer tube. Then 3 ml of uncontaminated blood are sucked up and the syringe is ed with a transparent "cannula", made of plexiss and a piece of polyethylene tubing. In a erculin syringe, the tip of which has been molten to a graduated glass capillary ending in a fine (*e. g.* a *Scholander-Roughton* pipette drawn out a fine tip) a gas bubble of about 5 mm^3 is meased between columns of 0.9 per cent NaCl solution equilibrated at the same gas tension as expected in the blood. Connection is now made between the plastic cannula and the tip of the graduated capillary, and the air bubble is pushed into the arterial blood in the glass syringe (Fig. 1). A few drops of blood are spilled out through the cannula, which is sealed by means of a short piece of copper wire.

The glass syringe, containing about 3 ml of arterial blood, an air bubble of about 5 mm^3 and a small drop of mercury for mixing, is then rotated mechanically at a regulated room temperature of about 37° C for 10—15 minutes.

The analysis of the bubble is performed in an apparatus as shown in Fig. 2. It consists — as Krogh's original apparatus, — of a glass capillary of 0.25 mm bore. One end of the capillary is bent at a right angle and smoothly widened to form a small cup. The other end is fitted with a water-filled chamber, into which a screw projects. The capillary is divided in 100 equal parts and by interpolation can be read to $^1/_{10}$ of a part. The measuring capillary is surrounded by a water jacket and mounted horizontally in such a way that it can be rotated around a longitudinal axis so that the cup

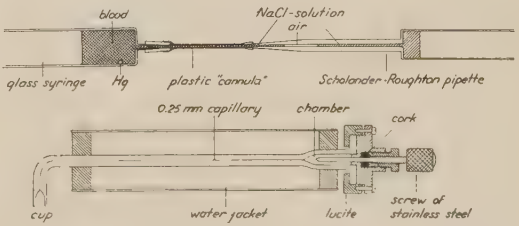

Fig. 1. (top) Transfer of air bubble from pipette to syringe.

Fig. 2. (below) Micro gas analyzer.

can be turned upwards when fluids are added, downwards when the air bubble is handled. The transfer of the bubble to the analyzer and the analysis of its composition is carried out at a room temperature of about 37° C.

The transfer of the air bubble from the equilibration syringe to the analyzer is carried out as follows: The cup of the analyzer is filled with water equilibrated with air at O_2 — and CO_2 — tensions approximately equal to those expected in the blood, and part of this water is sucked into the measuring capillary by means of the screw. The remainder of the water in the cup is then sucked off, and the cup is filled with blood from the syringe after flushing of the cannula with a few drops. The analyzer is now turned 180 degrees so that the cup points down. The equilibration syringe is held vertically with the cannula upwards so that the air bubble flows to the entrance of the cannula. By careful manipulation of the plunger it is forced halfway into the cannula, and then the tip of the polyethylene cannula is pushed up through the blood and placed near the bottom of the inverted cup of the analyzer. The air bubble is pushed out into the cup, from where it is immediately drawn into the measuring capillary by means of the screw and measured. The analyzer is now turned so that the opening of the cup points up, the blood is sucked off and the cup is filled with KOH-solution. With the cup pointing down again the air bubble is screwed a few times almost completely down into the KOH-solution and then back into the capillary for measuring. The cup is now emptied and filled anew with O_2-absorbent and the foregoing procedure repeated. O_2 is usually absorbed after 10—12 movements. Care is taken always to keep the last "tail" of the air bubble in the capillary, lest it flow out in the cup and absorbent enter the capillary. This often causes the bubble to break up in smaller parts. Should this nevertheless happen, a piece of fishgut, dipped in caprylic alcohol, can be brought in contact with the bubble, which then usually unites again. Caprylic alcohol in the measuring capillary should be avoided as it is apt to break the column of air. Between analyses the analyzer is rinsed with distilled water several times, the last time with water equilibrated at the expected gas tensions. Over night the capillary is first rinsed with a detergent, then left with distilled water in the hot room. Rinsing with *e. g.* sulphuric acid should be done only when absolutely necessary, and afterwards very thorough rinsing with weak alkali, detergent and water should be performed.

TESTS OF THE ACCURACY OF THE METHOD

In order to test the different steps of the method a series of determinations of known gas tensions was performed.

1) An air bubble (about 5 mm³) of known composition was introduced into the analyzer with *water* equilibrated at the same gas tensions and analyzed. The differences from the known tensions (pCO_2 35 mm and pO_2 110 mm) were averaged and the standard deviations calculated.

The result was:

ΔpCO_2: —1.2 mm	ΔpO_2: +1.2 mm
d = ±1.6	d = ±1.0 mm
n = 13	n = 13

2) An air bubble of known composition was introduced into the analyzer with *blood* equilibrated at the same gas tensions and analyzed.

Result:

ΔpCO_2: —1.5 mm	ΔpO_2: +1.5 mm
d = ±2.7 mm	d = ±1.3 mm
n = 30	n = 30

3) A bubble of *atmospheric air* was introduced into a syringe containing blood equilibrated at ordinary room temperature with a gas mixture of known composition (about 5 per cent CO_2 and 13 per cent O_2) and rotated for 10—15 minutes at room temperature. It was then transferred to the analyzer as described and analyzed.

Result:

ΔpCO_2: 0 mm	ΔpO_2: +0.1 mm
d = ±2.4 mm	d = ±1.9 mm
n = 22	n = 22

) A bubble of approximately 100 per t N_2 was rotated with blood equilibrated a pCO_2 of 35 mm and a pO_2 of 110 mm about 15 minutes, transferred to the ana- r and analyzed.

Result:

ΔpCO_2: —1.3 mm	ΔpO_2: —3.6 mm
d = ±2.6 mm	d = ±3.6 mm
n = 10	n = 10

Krogh (1908) pointed out that the total ssure of the gases in arterial blood was er than that of the atmosphere and of the eolar air. He showed that as a consequence this a bubble with a total pressure higher n that of the blood gases would undergo inking and that the equilibrium reached uld be a "sliding equilibrium". In Krogh's rotonometer a special pressure regulator usted the pressure in the bubble to that of blood gases so that no such shrinking k place. In special experiments Krogh nonstrated that a difference in total pres- es made itself most apparent on the pN_2, ch less on the pO_2 and was negligible on pCO_2. A difference in total pressures of mm Hg (1 per cent of an atmosphere) ulted in an error on the pO_2 of less than m Hg. The actual pressure difference be- en arterial and alveolar gases is for the in part due to the alveloar-arterial pO_2 erence which is found to be 5 to 25 mm by different authors. If a negative al- lar-arterial pN_2-difference of 8 to 16 mm exists, as found by Canfield & Rahn (1957) and Aksnes & Rahn (1957) the total pressure difference will be reduced to a value which, according to Krogh, should cause but a slight error on the pO_2 and none on the pCO_2 as determined by the bubble method without pressure regulation. In order to see if the present method also gives only slight errors with moderate differences in the total pressures, a series of control experiments was performed in which the total pressure of the blood gases was adjusted to 20 or 30 mm Hg below barometric pressure.

5) A bubble of atmospheric air was rotated for 10—15 minutes in blood equilibrated with a gas mixture of known composition at a total pressure *20 to 30 mm Hg below ambient barometric pressure*. The bubble was analyzed as usual and the partial pressure compared to the known gas tensions of the blood.

Result:

ΔpCO_2: +1.0 mm	ΔpO_2: +2.2 mm
d = ±2.4 mm	d = ±2.7 mm
n = 26	n = 26

As the results show, both the pCO_2 and the pO_2 came out too high. For the pO_2 Krogh found that when the total pressure was 39 mm Hg lower in the dissolved gases than in the bubble, the pO_2 was found 5.4 mm Hg too high. In the present experiments it is 2.2 ± 0.5 mm Hg too high. The difference between this average, and the average in series 3): 0.1 ± 0.4 mm Hg is, however, not statistically significant. For pressure differences as low as those to be expected in normal arterial blood, the error caused by a "sliding equilibrium" therefore must be slight and indistinguishable from the systematic error inherent in the method.

In the above mentioned tests, equilibration and analysis were performed at ordinary room temperature. Attempts were made to equilibrate the bubble at 37° C and to transfer it as quickly as possible to the analyzer which was kept at room temperature. The drop in pO_2 was considerable that it was deemed necessary to perform the transfer and the analysis of the gas bubble also at 37° C in a thermoregulated room. In order to simulate experimental conditions as closely as possible the arterialized blood was stored on ice in the equilibrating syringes for 20 to 40 minutes before it was rewarmed in water at 37° C for 8—10 minutes, the bubble was introduced and the rotation with consequent transfer and analysis at 37° C room temperature were begun. Lambertsen, Bunce, Drabkin & Schmidt (1952) found that addition of fluoride and cyanide to the blood had no measurable effect on the stability of the gas tensions. In earlier experiments we have found (Asmussen & Nielsen, 1956) that fluoride prevents a drop in pH of blood stored for 2 hours at about 37° C. Under the present experimental conditions, where the blood was kept at 37° C for only 20 to 30 minutes, our results confirm those of Lambertsen *et al.* as the following test shows.

6) An air bubble was equilibrated with blood of known gas tensions at 37° C and analyzed at the same temperature. The blood was treated with fluoride + cyanide, fluoride only, or left untreated.

Results:

Fluoride + cyanide

ΔpCO_2: —1.9 mm	ΔpO_2: +0.3 mm
d = ±1.4 mm	d = ±1.0 mm
n = 13	n = 13

Fluoride

ΔpCO_2: —4.5 mm	ΔpO_2: —0.3 mm
n = 6	n = 6

None

ΔpCO_2: +0.1 mm	ΔpO_2: —0.3 mm
d = ±1.4 mm	d = ±1.3 mm
n = 16	n = 16

In the following experiments, therefore, no chemicals except heparin were added to the blood.

7) Blood was equilibrated with a gas mixture of known composition at 37° C, anaerobically drawn into the equilibrating syringe which contained a drop of mercury, stored on ice for 20 minutes, rewarmed in water to 37° C, equipped with a bubble of atmospheric air and rotated for 10—15 minutes. The bubble was transferred to the analyzer and analyzed at 37° C.

Result:

ΔpCO_2: +1.0 mm	ΔpO_2: —0.8 mm
d = ±2.01 mm	d = ±1.97 mm
n = 55	n = 55

COMMENTS

It appears from the preceeding that, for measurements of arterial CO_2 and O_2 tension, the described method gives reliable results when the arterial tensions are in the vicinity of the normal (pCO_2 about 40 mm, pO_2 about 90 mm) when a bubble of atmospheric air is used. Even when the bubble is pure N_2 or when the total gas pressure in the blood is 20—30 mm Hg below the total pressure of the bubble, the results do not deviate more than a few millimeters on the pO_2 and less on the pCO_2. Under the described circum-

nces fluoride and cyanide need not be ded to the blood. A close control of tem- ature — to within 0.1° C — is necessary. rrections for temperature differences were de by means of factors from Brown & Hill 922) for pO_2 and from Brewin, Gould, shat & Neil (1955) for pCO_2. The nomo- ms of Bradley, Stupfel & Severinghaus 956) may also be used and give the same ults. Some special precautions must be ntioned: The drop of mercury in the equi- rating syringe is necessary to obtain equi- rium. All glass and mercury that come o contact with the blood must be comple- y free from traces of acid, lest the pCO_2 comes too high. The capillary of the ana- er must be kept clean. Rinsing can be per- med with distilled water between analyses; en a more thorough cleansing is necessary, ergent may be used.

The results seem to indicate that CO_2 ten- ns are determined about 1 mm too high, hereas the O_2 tensions are about 1 mm too v. The systematic erorr and the standard viation are of the same magnitude as those nd by Lambertsen *et al.* (1952) and Filley *al.* (1954, using modifications of Riley's hnique.

SUMMARY

A description is given of a method for ect determinations of oxygen and carbon xide tensions in arterial blood, based on bubble method of Krogh (1908) and the difications introduced by Riley *et al.* 945), Lambertsen *et al.* (1952) and others. The bubble, about 5 mm^3, is equilibrated arately in 3 ml of blood by rotation at ° C for 10—15 minutes and then trans- ferred to the micro gas analyzer. Tests with air bubbles of various composition were made in blood at O_2—tensions around 90 mm Hg and CO_2 tensions about 40 mm Hg. Systematic errors were $+ 1 \pm 2$ mm Hg for pCO_2 and -1 ± 2 mm Hg for pO_2 when the bubble was atmospheric air.

REFERENCES

Aksnes, E. & Rahn, H.: Measurement of total gas pressure in blood. J. Appl. Physiol. *10,* 173, 1957.

Asmussen, E. & Nielsen, M.: Physiological dead space and alveolar gas pressure at rest and during muscular exercise. Acta Physiol. Scandinav. *38,* 1, 1956.

Bradley, A. F., Stupfel, M. & Severinghaus, J. W.: Effect of temperature on P_{CO_2} and P_{O_2} of blood in vitro. J. Appl. Physiol. *9,* 201, 1956.

Brewin, E. G., Gould, R. P., Nashat, F. S. & Neil, E.: An investigation of problems of acid-base equilibrium in hypothermia. Guy's Hospital Reports *104,* 177, 1955.

Brown, W. E. L. & Hill, A. V. (1922) Cit. in Dill, D. B., Graybiel, A., Hurtado, A. & Taquini, A. C.: Der Gasaustasch in den Lungen im Alter. Ztschr. f. Altersforschung *2,* 1, 1940.

Canfield, R. E. & Rahn, H.: Arterial-alveolar N_2 gas pressure differences due to ventilation-perfusion variations. J. Appl. Physiol. *10,* 165, 1957.

Krogh, A.: Some new methods for the tonometric determination of gas-tensions in fluids. Skandinav. Arch. Physiol. *20,* 259, 1908.

Lambertsen, C. J., Bunce, P. L., Drabkin, D. L. & Schmidt, C. F.: Relationship of oxygen tension to hemoglobin oxygen saturation in the arterial blood of normal men. J. Appl. Physiol. *4,* 873, 1952.

Riley, R. L., Proemmel, D. D. & Franke, R. E.: A direct method for determination of oxygen and carbon dioxide tensions in blood. J. Biol. Chem. *161,* 621, 1945.

Scandinav. J. Clin. & Lab. Investigation 272—277, 10, 1958.

A MODIFICATION OF THE ALKALINE COMBUSTION METHOD FOR THE DETERMINATION OF PROTEIN-BOUND IODINE IN SERUM*

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The procedure presented below is a modification of the method for protein-bound iodine determination introduced by Barker, Humprey & Soley in 1951, which was formerly used in our laboratory. The main modifications in the procedure are the dialysis of the serum to remove the inorganic iodine, the drying and incineration with potassium hydroxide in nickel crucibles, and the extraction of the ash with strongly alkaline medium (Vilkki, 1956). The advantages offered by alkaline extraction have recently been pointed out also by other workers (Grossman & Grossman, 1955; Van Slyke, 1957).

The new modification has now been employed over three years and has been found practical in routine analytical work. It seems to be less sensitive to external iodine contamination, yet it yields results as accurate as the results obtained by the original method. The determinations are easily carried out by a laboratory technician. When the time required for the dialysis is disregarded, about 20 duplicate samples can be analyzed during an 8-hour workday by one worker.

* Read at the International Congress on Clinical Chemistry, Stockholm, August 19—23, 1957.

PRACTICAL CONSIDERATIONS

1. Instead of precipitating the serum proteins with zinc salt and performing numerous washings, the inorganic iodine is removed from the samples by dialysis in cellophane bags under ordinary tap water. The water must not contain more than about 10 micrograms of iodine per litre. The dialysis must be of at least 12 hours' duration.

2. The bags and their dialyzed contents are placed in nickel crucibles and the alkaline incineration reagent added. The reagent contains potassium hydroxide as the alkali and zinc sulphate as the catalyst. The drying is rapidly carried out in a furnace at a temperature of about 150° C. A temperature as high as this can be used since the evaporating area in the crucible is large. The samples do not froth if the ventilation in the furnace is adequate during evaporation. During the incineration an even temperature of 600° C in the furnace is preferable. The ventilation holes in the furnace must be closed at this stage.

3. The resulting ash is suspended in neutral arsenite solution and centrifuged. It is important that the solution be kept strongly alkaline during the whole dissolving proce

e. The alkaline reaction ensures an easy trifugation which yields a water-clear ernatant. Heavy metals which may posly interfere in the photometric determina- are also precipitated as carbonates from solution. The alkalinity of the solution seems to protect the iodide from oxidan and prevent its volatilization during the solution. The alkalinity is maintained until clear supernatant solution has been transred to the photometer tube, in which it is dified with a sulphuric-hydrochloric acid gent. The carbon dioxide liberated prots the contents of the tube from oxidation. . The catalytic action of iodide ion on reduction of quadrivalent cerium ion fers from Barker's method only in that concentrations of acid, chloride and arite ions are higher and the concentration cerium ion is lower in the new modifican (Lein & Schwarz, 1951). The rate of dispearance of the yellow color is determined taking two photometer readings, one six nutes and the other 12 minutes after the dition of the cerium reagent. The interval tween the readings does not necessarily ve to be 6 minutes, and can be altered fit the prevailing conditions. Also the nperature at which the color reaction is rried out, usually 40° C, can be lowered a slower reaction is desired. By these ans the per cent transmission can be justed to remain between 20 and 65 during recording. The kinetic recording of the luction rate of cerium has been retained hough two methods yielding a stable ium color were also tested (Rogin & bravcic, 1953; Grossman & Grossman, 55). The stable color increased the accuy of the photometer scale reading, but often gave erroneous results, probably owing to the presence of foreign reducing agents. When the catalytic activity of the iodide ion is determined kinetically, these agents seem to cause a rapid initial reduction but do not interfere with the rate of reduction at a later stage when an accurate measurement of the rate can be made.

To check the recovery of iodine in the analysis, each analysis is carried out on duplicate samples, to one of which a standard amount of iodine is added after dialysis. The level of results in each series is thus effectively controlled.

METHOD

Reagents

Water. Redistilled water, controlled to be low in iodine.

Incineration reagent. 70.1 g of potassium hydroxide and 25.0 g of zinc sulphate are separately dissolved in 250 ml of water. Equal volumes of the two solutions are mixed immediately before use.

Potassium arsenite. A. Stock solution, 0.2 *N*. 4.95 g of arsenic trioxide are dissolved in 100 ml of boiling water to which 6 ml of 5 *N* potassium hydroxide have been added. After dilution to approximately 300 ml in a 500-ml volumetric flask, the solution is neutralized with 4.3 ml of 7 *N* sulphuric-hydrochloric acid reagent, after which the flask is filled to the mark. B. For actual use, two volumes of the stock solution are diluted with three volumes of water to give a 0.08 *N* solution.

Sulphuric-hydrochloric acid reagent, 7 N. 98 ml of concentrated sulphuric acid are diluted to 300 ml with water, 50 g of sodium chloride are added, and the solution is diluted to 500 ml.

Ceric ammonium sulphate, 0.01 N. 3.17 g of ceric ammonium sulphate are dissolved in approximately 300 ml of water. The solution is acidified with sulphuric acid (84 ml of concentrated acid) to obtain a solution which, after dilution to 500 ml, has a final acidity of 6 *N*.

Standard iodide solution. A. Stock solution.

0.0645 g of potassium iodide is dissolved in 500 ml of water. The titer of this solution is determined by oxidizing the iodide in a 5-ml sample to iodic acid with potassium permanganate and titrating the iodine liberated after adding potassium iodide with standard sodium thiosulphate solution (the normality of the latter is determined against potassium iodate). A 5-ml volume of the above solution is made alkaline by adding one drop of 5 *N* potassium hydroxide in a 500-ml volumetric flask and the flask is filled to the mark with water. This stock solution is stored in a refrigerator at 4° C. B. For actual use, 2 ml of the stock solution are diluted to 50 ml anew for each series of analyses. The iodine content of this solution is 0.040 microgram per ml.

All reagents used must be of analytical grade. The iodine contents of the reagents have been so low that further purification has not been found necessary. The blank value is nevertheless determined for the reagents in each series of analyses.

Laboratory equipment

Dialyzer tubing. ¾ inch standard cellophane tubing.

Nickel crucibles. These are of pure nickel, 40 mm in height and in diameter, and about 20 ml in volume.

Combustion furnace. Heraeus muffle furnace, the temperature of which can be adjusted to 150° C and 600° C. The crucibles can be placed inside in two layers separated from each other by brass gauze, the maximum number of crucibles being 50.

Centrifuge tubes. Conical standard centrifuge tubes, volume about 15 ml.

Photometer. Beckman spectrophotometer Model B fitted for standard cylindrical cuvettes.

Water thermostat. Electrically controlled to 40 ± 0.1° C.

Analytical procedure

Dialysis

Pieces about 6 cm in length are cut from dialyzer tubing and immersed in water. The wet pieces are tied at one end with cotton thread and an aluminum number plate is attached to the knot. The tubes, open at one end, are placed in an ordinary test tube rack. One ml of the serum to be analysed is pipet ted into each of two bags and the bags are tied a the upper ends. In each series, two bags containin one ml of water instead of serum are also include to obtain the blank value. The bags are dialyze under running tap water at least 12 hours in vessel large enough to prevent the bags from touch ing each other.

Incineration

The bags are transferred to nickel crucibles an opened by cutting through the knot carrying th number plate. 2 ml of incineration reagent are adde and mixed with the contents of the opened bag wit a glass rod. To one of the two crucibles for eac serum sample, one ml of standard iodine solutio is added. The crucibles are transferred to a muffl furnace, temperature about 150° C, where they ar allowed to remain until the water has completely eva porated (2—3 hours). After the samples have dried the temperature of the furnace is raised to 600° C where it is kept for two hours.

Dissolving the ash

After the crucibles have cooled, exactly 5 ml o potassium arsenite solution are added. The as partly dissolves in the reagent solution and th remainder is readily suspended by shaking th crucible. The solution is strongly alkaline at thi stage owing to the potassium carbonate forme from the potassium hydroxide. The suspension i poured from the crucible into a centrifuge tub and centrifuged about 10 minutes at approximatel 1500 r.p.m. The suspended matter (carbon and in soluble carbonates) separates to the bottom of th tube. The supernatant layer containing the iodide i solution is water clear.

Color reaction

Exactly 3 ml of the clear layer are pipetted int a photometer tube, care being taken to avoid trans ferring any solid residue. One ml of 7 *N* sulphuric hydrochloric acid reagent is added carefully to th tube to avoid frothing.

The photometer tubes, no more than 18 to series, are transferred to a water thermostat a

C for at least 15 minutes. A test tube contain- the required volume of cerium solution is also ed in the thermostat for the same period.

ne ml of cerium solution is added to each of hotometer tubes in the thermostat in succession 0-second intervals as read from a stop watch. r each addition the tube in question is vigor- y shaken and replaced in the water bath. The reagent still effects an appreciable frothing h facilitates the mixing.

ix minutes after the first addition of cerium ent, the per cent transmission of the first is determined at 4200 Å against distilled water. readings are taken similarly at 20-second inter- for the remaining tubes in succession, i. e. in case after six minutes have elapsed from the tion of the cerium solution. The transmission ach tube is read again after exactly six minutes elapsed from the first reading. If the second smission reading exceeds 65 per cent, the ana- must be repeated on a smaller sample.

Calculation of results

he difference (ΔT) between the photometer lings taken at the beginning and at the end of 6-minute interval is calculated for each sample. s difference is a measure of the rate of the t-order reaction $Ce^{IV} \rightarrow Ce^{III}$ which is, under specified conditions, with sufficient accuracy ctly proportional to the iodine content of the ple. When the iodine content (0.0—20.0 micro- ms per 100 ml) is plotted against the difference er cent transmission ΔT (0.0—30.0 per cent), raight line results which intersects the horizontal at the point PBI = 0, the corresponding ΔT ue for which is given by the photometric value the blank sample containing only the reagents T_0) (Fig. 1). The slope of the line gives the ease in ΔT due to the amount of iodine (0.04 rogram) added to the duplicate. This increase alculated by subtracting the ΔT value for the ple from the ΔT value for the corresponding licate containing standard iodine; the average ease for the combustion series ($\Delta T_{m.04}$) is used n drawing the plot. The average value gives a t on the linear plot corresponding to an iodine ent of 4.0 micrograms per 100 ml ($\Delta T_{4.0} =$

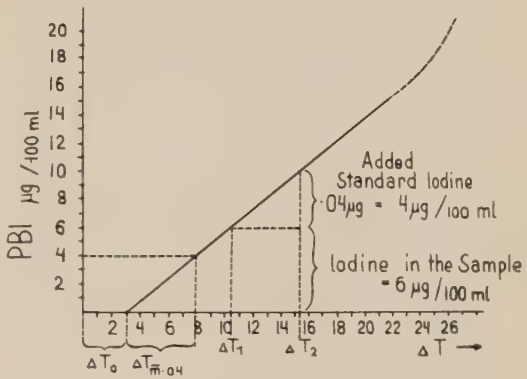

Fig. 1. The standard plot used for calculation of results. The values of protein-bound iodine (PBI) are plotted against the difference (ΔT) between the per cent transmission readings taken at six-minute intervals.

$\Delta T_0 + \Delta T_{m.04}$). The linear plot can be drawn when the two values, ΔT_0 and $\Delta T_{4.0}$, are known and the iodine content of a sample can be read from the plot when the value of ΔT for the sample has been determined.

The result can also be calculated with the aid of the equation for the linear plot

$$\text{iodine in micrograms} = 0.04 \frac{n(\Delta T_1 - \Delta T_0)}{\Sigma(\Delta T_2 - \Delta T_1)}$$

where

ΔT_1 = difference in the photometer readings for the sample.

ΔT_2 = difference in the photometer readings for the duplicate to which 0.04 microgram of iodine had been added.

ΔT_0 = difference in the photometer readings for the blank.

n = number of duplicate analyses in the series.

RESULTS

The effectiveness of the dialysis was studied on a series of twenty samples of the same serum, to ten of which 0.08 microgram of standard iodide had been added before the dialysis. (In the final result this addition corresponds to 8 micrograms of PBI in

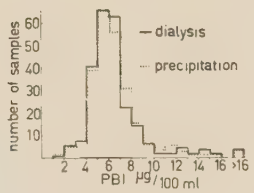

Fig. 2. Frequency distribution of PBI values for 500 blood samples. 250 of these samples were analysed by the precipitation method (dotted line), and the other 250 using the dialysis method (solid line).

100 ml of serum.) PBI determinations gave a mean value of 4.9 ± 0.25 micrograms for the serum alone and 4.8 ± 0.21 micrograms for the serum to which the iodide had been added. This result shows that the dialysis efficiently extracts all inorganic iodide.

The results for 500 blood samples from 500 patients are shown distributed according to their PBI values in Fig. 2. 250 samples were analyzed with the dialysis method and 250 with the protein precipitation method. In all other respects the analytical procedure was the same in the two series. No significant differences are noted between the distributions of PBI in the two series.

A further series of 20 serum samples were analysed by both methods simultaneously. The PBI values obtained (Table I) after dialysis were 0.26 microgram per 100 ml higher on the average than those obtained after precipitation and washing. This is not a significant difference, although it reveals a tendency for the dialysis method to give slightly higher values.

The error of the dialysis method, the standard deviation of a single determination calculated from the differences between duplicate analyses, is ± 0.4 microgram of iodine per 100 ml of serum.

Table I. *PBI values in micrograms pe 100 ml serum for 20 samples determine simultaneously by dialysis and precipitatio methods.*

No.	Dial.	Precip.	Diff.	No.	Dial.	Precip.	Diff.
1.	5.4	5.8	—0.4	11.	5.7	6.2	—0.
2.	5.2	5.1	+0.1	12.	6.7	5.8	+0.
3.	5.4	6.1	—0.7	13.	5.7	5.7	0
4.	5.8	5.6	+0.2	14.	5.0	4.6	+0.
5.	6.8	6.0	+0.8	15.	6.5	4.5	+2.
6.	5.5	4.2	+1.3	16.	5.8	5.8	0
7.	12.4	11.5	+0.9	17.	4.8	5.4	—0.
8.	4.8	4.8	0	18.	5.6	5.4	+0.
9.	6.0	5.5	+0.5	19.	4.6	5.0	—0.
10.	7.7	7.5	+0.2	20.	4.9	4.6	+0.
		Average			6.0	5.8	+0.2

The method has a wide application also i the determination of iodine in other sub stances than blood. It is suitable for th determination of iodine in all kinds of tis sues and foodstuffs. For these analyses, n further modification is necessary except tha the dialysis step is omitted. The standardiza tion of the method and its application in foo analysis have been extensively discussed i a previous paper of the writer (Vilkki,1956

SUMMARY

A modification of the alkaline incineratio method for the analysis of protein-boun serum iodine is described. The main di ferences from the usual procedures are:

1. Dialysis instead of protein precipit tion is used to eliminate inorganic iodi from the samples.

2. The samples are dried and incinerate with potassium hydroxide in nickel crucible

3. The ash is extracted with strongly a kaline medium.

. To control the level of the results, re-
ery of added iodide is determined in each
ies of analyses.

REFERENCES

ker, S. B., Humprey, M. J. & Soley, M. H.: The clinical determination of protein-bound iodine. J. Clin. Investigation *30,* 55, 1951.

ssman, A. & Grossman, G. F.: Protein-bound iodine by alkaline incineration and a method for producing a stable cerate colour. J. Clin. Endocrinol.*15,* 354, 1955.

Lein, A. & Schwartz, N.: Ceric-sulphate — arsenious acid reaction in microdetermination of iodine. Anal. Chem. *23,* 1507, 1951.

Rogin, B. & Dubravcic, M.: Micro-determination of iodides by arresting the catalytic reduction by iodides of ceric ions. Analyst *78,* 594, 1953.

Van Slyke, D. D.: Alkaline incineration methods for determination of protein-bound iodine in serum. Paper read at Int. Congress of Clin. Chem., Stockholm, 1957.

Vilkki, P.: Iodine in the Finnish diet and its relation to goitre incidence. Ann. Acad. Scient. Fennicae, Series *A,* II., Chemica, 71, 1956.

Scandinav. J. Clin. & Lab. Investigation 278—282, 10, 1958.

STUDIES ON INTRINSIC FACTOR AND PERNICIOUS ANEMIA

I. ORAL UPTAKE OF VITAMIN B_{12} IN PERNICIOUS ANEMIA WITH INCREASING DOSES OF AN INTRINSIC FACTOR CONCENTRATE

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The purpose of the present study was to find the relationship between intrinsic factor (IF) activity and vitamin B_{12}-binding capacity for hog pylorus concentrates of considerably different purity and for different fractions of such concentrates. The intention was to show whether or not the B_{12}-binding capacity of hog pylorus concentrates could be used for expressing their IF activity, and whether or not the B_{12}-binding capacity and the IF activity can be ascribed to the same component or components.[1]

Already here it must be emphasized that the B_{12}-binding capacity, if at all present for IF as such, is not specific for IF, as this property is proved also for certain other known compounds and animal materials without IF activity.[1] Consequently, one necessary condition for expressing IF activity in terms of B_{12}-binding capacity must be that no such compounds have been added.

In this first part of the study it will be shown how pernicious anemia (PA) patients responded to different amounts of a commercial IF concentrate, primarily in order to establish the preferable dosage for the subsequent investigations.

[1] For a comprehensive summary and discussion of earlier studies on this problem the reader is referred to Gräsbeck and Wijmenga (1956).

MATERIAL

The IF preparation used in this study was certain batch of the commercial material from ho pyloric mucosa used in Bendogen tablets (A/S Ga Copenhagen, and A/B Kabi, Stockholm). Bendoge tablets (Kabi) contain 10 μg vitamin B_{12} togethe with about 50 mg (10,000 coli units, see below) of th IF material. The batch of IF material used is her referred to as "IF reference standard" and has B_{12}-binding capacity of 202 coli units per mg.

The PA patients used for the tests were selecte from available patients at the Central County Ho pitals of Eskilstuna and Falköping, Sweden. The all were patients with well-established PA, none o which was known to be clinically refractory to or treatment.

METHODS

1. The *B_{12}-binding capacity* was determined a cording to a method in principle developed by Hof Jørgensen, Skouby & Gad Andresen (1952), mod fied and simplified by Dr. Niels Nielsen, head o the microbiological laboratory of A/B Kabi, Stock holm. The B_{12}-binding capacity is expressed in co units (CU), 1,000 CU being defined as the amou of the IF preparation capable of making 1 μg vit min B_{12} unavailable to E. coli.

The method is in principle carried out by additi of an excess of vitamin B_{12} to the IF preparati to be assayed, and determination of free vitam B_{12} by means of a strain of E. coli. The bou vitamin is obtained as the difference between tot and free B_{12}.

An aqueous solution of the IF preparation wi an estimated concentration of about 1,000 CU/ml prepared, and three 2 ml portions of this soluti

mixed with 2 ml of B_{12} solutions containing 3.3, and 5.0 μg B_{12}. The amount of B_{12} added is about twice the amount bound by the IF prestion. As a precautionary measure the solutions allowed to stand for 1 hour at 25° C, although formation of IF-B_{12} complex is assumed to take e very rapidly.

he amount of free B_{12} is determined in the three tions with E. coli 113-3 as test organism (Davis Mingioli, 1950). The assay is made as a plate The amount of bound B_{12} is calculated as difnce. The three solutions give essentially the e values for bound B_{12}. This determination is ably carried out in duplicate or triplicate, and mean value is calculated.

F solutions weaker than 1,000 CU/ml are asd by addition of correspondingly smaller unts of B_{12}. In this case the more sensitive tube is needed for the B_{12} determination. Thus IF tions with as little as 10 CU/ml can be assayed. his procedure has repeatedly been shown to essentially the same values for B_{12}-binding acity as Hoff-Jørgensen's method. It has also compared with the dialysis method (Rosenn, Woodbury & Reisner, 1954), utilizing radioe vitamin B_{12}. Also this method gives comable results.

. The *IF activity* was determined by measureit of the urinary radioactivity excretion accordto Schilling. The radioactivity determinations e all carried out at the Central Laboratory, ilstuna, with a scintillation counter.

μg B_{12} with 0.25 microcurie B_{12}-Co^{60} (supplied Merck & Co.) was given on a fasting stomach he morning, alone or along with an IF dose. B_{12} dose was given in about 80 ml water, and IF preparation was suspended in the solution ediately before the dose was taken. Two hours r 1,000 μg of non-radioactive vitamin B_{12} were inistered intramuscularly. The urine was coled for the first and in most cases also for the nd 24-hour period after the oral dose. A second ction of 1,000 μg B_{12} was given 24 hours after oral dose, and at least 3 days elapsed between doses of radioactive B_{12} to the same patient. mples of the collected urines were concentrated imes by evaporation in vacuum and counted in l portions in a Tracerlab well-type crystal. By comparison with a simultaneously counted standard of the radioactive B_{12} used, the urinary excretion of radioactivity was calculated as percentage of the given dose.

EXPERIMENTAL

The Schilling test was first carried out on a number of PA and non-PA subjects with radioactive B_{12} alone, in order to make sure that the method worked satisfactorily in our hands.

As expected, very great differences in urinary excretion were found in PA and in hematologically normal subjects (Fig. 1). Of the non-PA cases those with achlorhydria show on an average slightly lower values than those without achlorhydria; the difference is small but significant at the 0.1—0.5 per cent level (** significance). The cause of this difference may be that some achlorhydria patients are developing a PA, yet without clinical symptoms. Two cases of nontropical sprue fall in the same range as PA.

The Schilling test was then carried out on a number of PA patients with the use of 1 μg radioactive vitamin B_{12} and varying amounts of IF reference standard ranging from zero to 100,000 CU. The results are visualized in Fig. 2.

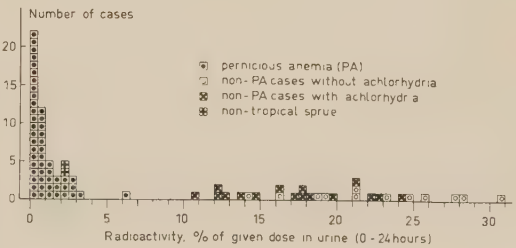

Fig. 1. Urinary radioactivity excretion after 1 μg B_{12}-Co^{60} without intrinsic factor (Schilling test). Demonstration of difference between pernicious anemia and other subjects.

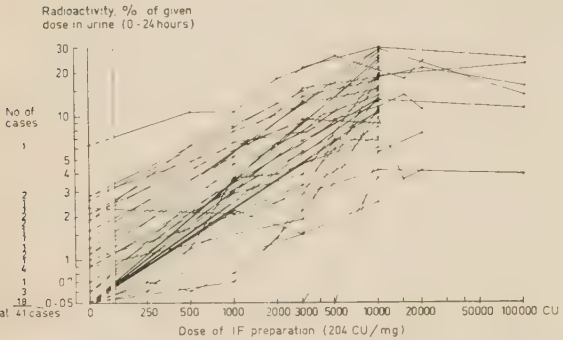

Fig. 2. Demonstration of influence of intrinsic factor dose on oral B_{12} uptake in pernicious anemia. Schilling test with 1 μg B_{12}-Co^{60} in 41 subjects.

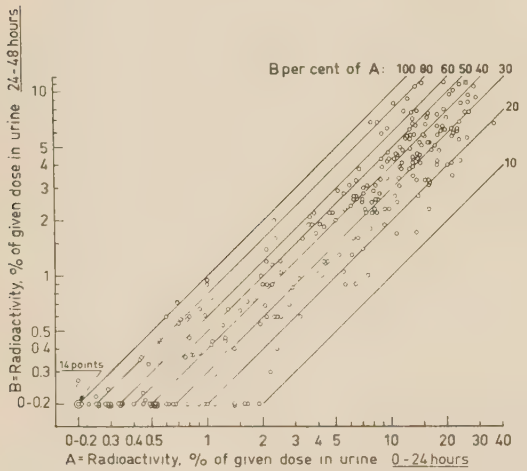
B per cent of A: 100 80 60 50 40 30
20
10
B=Radioactivity, % of given dose in urine 24-48 hours
14 points
A=Radioactivity, % of given dose in urine 0-24 hours

Fig. 3. Urinary radioactivity excretion test according to Schilling. 280 comparisons of excretion during first and second day after dose.

It may be worth mentioning that only values for 24-hour urine have been recorded here. The same general conclusions can be drawn from measurements made on 48-hour urine, although collecting of urine for 48 hours naturally results in higher absolute excretion values. On an average 35 per cent higher levels are obtained from 0—48 hour urine than from 0—24 hour urine, if 24 hour values below 2 per cent are excluded (Fig. 3). In a material comprising 280 comparisons between 0—24 and 24—48 hour urine the 0—24 hour excretion value was above 2 per cent in 183 cases. 155 of these (85 per cent) had a 24—48 hour value in the range of 20—60 per cent of the 0—24 hour value. Consequently, our opinion is that for routine tests excretion values based only on 24-hour urine are in general quite acceptable.

RESULTS AND DISCUSSION

Fig. 2 shows the influence of varying doses of our IF reference standard on the urinary radioactivity excretion, when given to PA subjects with an oral dose of 1 μg radioactive vitamin B_{12}. The following general conclusions can be drawn.

Significant increase in B_{12} uptake is obtained by IF doses higher than approximately 500 CU.

A dose of 1,000 CU, which is "equivalent" to the B_{12} dose given, in terms of binding capacity, is not sufficient for maximal response. The implications of this fact will be discussed elsewhere; it does not, however, exclude the possibility of a proportionality between the two effects.

The response increases most sharply in the range of approximately 1,000—3,000 CU.

Maximal response occurs when an IF dose of approximately 10,000 CU or more is given. The response from 10,000 CU is in most cases but not invariably comparable with that generally found with 1 μg B_{12} alone in healthy subjects.

It has been claimed that very high IF doses may result in impaired radioactivity excretion (Glass, 1955). Possible explana-

s for this could be: a) the presence of nhibitory factor in the IF preparation, he presence of vitamin B_{12} in the IF con- rate, which may dilute the radioactive given. In our material there seems to slight tendency to lower values from ,000 CU than from 10,000 CU. The dif- nce is, however, not statistically signi- nt. An assay of vitamin B_{12} in our IF rence standard showed that a possible tent of B_{12} (< 0.2 μg/g) is practically ligible compared with the B_{12} dose given, n if 100,000 CU are used.

s seen from Fig. 2, the individual re- nse to the same IF dose is highly dif- nt from one patient to another. This may ly be explained by the fact that a large erial always will comprise cases of PA lifferent stages of development. Thus in of the PA cases, giving as high a re- nse as 6.3 per cent excretion after B_{12} ne, the deficiency in B_{12} absorption was arently not fully developed when the test s carried out, although the clinical picture convincing. (One year later the value s still as high as 5.0 per cent.) On the er hand, some of the cases recorded in . 2 show markedly low values after mode- e and sometimes also after high IF doses. know that some PA cases (reported by lin, Berlin, Brante & Sjöberg, 1958, and recorded here) may become refractory treatment with commercial oral prepara- ns, and that such patients show very low retion values even after an IF dose of 000 CU. It is possible that the subjects Fig. 2 with low response figures are dually becoming refractory to oral treat- nt with B_{12} + IF. We hope to be able to ert to this question after studying a num- ber of such cases for some more time. It may be mentioned already here that most of the cases with low response shown in Fig. 2 had received oral B_{12}-IF treatment at least during some period earlier. We do not yet know, however, if a refractoriness will develop as a result of or independently of the oral treatment.

If the patients shown in Fig. 2 are divided into two groups, one comprising those excreting only 0.5 per cent or less, the other more than 0.5 per cent, after B_{12} without IF, the first group appears to excrete less than the second also after B_{12} + 10,000 CU of the IF preparation. The difference is, however, not definitely established (* significance). A further study of this seems to be well justified. Possibly the B_{12} absorption in PA patients with particularly low spontaneous absorption cannot be fully restored by hog's IF in doses adequate in other cases.

Because of the great differences in excretion for different PA patients it is probably unjustified to construct an "average response" curve showing the radioactivity excretion after various IF-doses in a "normal" case of PA. The construction of a mean curve in Fig. 2 has accordingly not been attempted.

The immediate purpose of the material collected in Fig. 2, however, was to establish the IF doses most suitable for the subsequent experiments with different IF concentrates. It was considered important to choose IF doses corresponding to a steep section of the response curve, when the activity of different IF preparations were to be compared, to secure as dependable results as possible. For this purpose standard doses of 1,000 and 3,000 CU were chosen.

In our further experiments with the purpose of studying the response in suspected refractory cases from a dose known to be highly effective in normally susceptible PA subjects, a dose of 10,000 CU of the IF reference standard was generally adopted.

The relationship between B_{12}-binding capacity, expressed in CU/mg, and IF activity determined according to Schilling, for IF concentrates of considerably different purity, will be reported in a subsequent paper.

SUMMARY

The urinary excretion test according to Schilling was carried out on a number of pernicious anemia cases with the use of 1 μg radioactive vitamin B_{12} together with a commercial intrinsic factor concentrate in amounts varying from zero to very high doses.

The results are discussed, above all with the view of establishing standard doses most suitable for comparative activity studies of different intrinsic factor concentrates.

REFERENCES

Berlin, R., Berlin, H., Brante, G. & Sjöberg, S-G.: Failures in long-term oral treatment of pernicious anemia with B_{12}-intrinsic factor preparations. Acta Med. Scandinav. *161,* 143, 1958.

Davis, B. D. & Mingioli, E. F.: Mutants of *Escherichia Coli* requiring methionine or vitamin B_{12}. J. Bact. *60,* 17, 1950.

Glass, G. B. J.: Biochemistry and physiology of Castle's intrinsic factor and its relationship to the metabolism of vitamin B_{12}. Rev. Hématol. *10,* 137, 1955.

Gräsbeck, R.: Studies on the vitamin B_{12}-binding principle and other biocolloids of human gastric juice. Acta Med. Scandinav. Suppl. 314, 1956.

Hoff-Jørgensen, E., Skouby, A. P. & Gad Andresen, J.: Microbiological standardization and clinical effect of purified preparations of intrinsic factor. Nord. Med. *48,* 1754, 1952.

Rosenblum, C., Woodbury, D. T. & Reisner, Jr., E. H.: The use of cobalt-60 labelled vitamin B_{12} for the evaluation of intrinsic factor activity. Radioisotope Conference (Oxford) 1954, p. 287.

Wijmenga, H. G.: Intrinsic factor and vitamin B_{12} binding substances. Vitamin B_{12} und Intrinsic Factor. 1. Europäisches Symposium, Hamburg 1956, p. 156. Enke, Stuttgart.

ndinav. J. Clin. & Lab. Investigation 283—289, 10, 1958.

A SIMPLE FORMULA FOR THE MENTAL ARITHMETIC OF THE HUMAN BODY SURFACE AREA

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In most countries the formula given by Bois & Du Bois (1916) is used for the culation of human body surface area:

$$A = \frac{W^{0.425} \times H^{0.725} \times 71.84}{10\,000} \quad (1)$$

here A = surface area in m², W = weight kg and H = height in cm).

When using a calculator based on this mula an unexpected relationship was nd in adults of about 160 cm height. For ese persons the simple formula

$$A = 1 + \frac{W}{100} \quad (2)$$

emed to be valid within certain limits.

Moreover, in other persons the two first cimals of the surface area values seemed deviate from the figures of the body weight proximately as much as the body height viated from 160 cm.

In order to study these observations the lowing calculations were performed, re-lting in a simple formula applicable to rface areas larger than 1.0 m² and suitable mental arithmetic (Formula 5).

CALCULATIONS

Validity of Formula 2:

First of all it was of interest to calculate the ghts at different weights when the Formula 2 es "exact" surface area values, in cases when

$$1 + \frac{W}{100} = \frac{W^{0.425} \times H^{0.725} \times 71.84}{10\,000} \quad (3)$$

The equation was solved for log H

$$= 1.38 \times \log (W+100) - 0.586 \times \log W + 0.1981 \quad (4)$$

Different figures for the weight (W) were inserted in Equation 4 and the height (H) was calculated. The results are listed in Table I.

As seen from the table the "ideal" height for the weight range 40—120 kg varies within certain narrow limits (156.3—165.2 cm), showing minimum values at 70—80 kg. The mean value of the weight range mentioned is close to 160 cm. A gradual deviation from this height will result in increasing errors of the surface area when using Formula 2. The magnitude of the errors will be shown in the following calculation.

Table I. *Body height at given body weights where the Formula 2, $A = 1 + \frac{W}{100}$, gives the same surface area as the formula of Du Bois & Du Bois*

Body weight kg	Body height cm	Body surface area m²
40	165.2	1.40
50	160.0	1.50
60	157.4	1.60
70	156.3	1.70
80	156.4	1.80
90	157.2	1.90
100	158.7	2.00
110	160.5	2.10
120	162.6	2.20
Mean	159.4	-

Table II. *The error in m² if the Formula 2, $A = 1 + \frac{W}{100}$, is used for the calculation of the surface area, instead of the formula of Du Bois & Du Bois.*
(The number of cases constituting Material A is indicated in brackets).

Height cm	130	140	150	160	170	180	190	200
ΔH	−30	-20	−10	± 0	+ 10	+ 20	+ 30	+ 40
Weight kg	Error in m²							
30	+ 0.26	+ 0.20	+ 0.15	+ 0.09	-	-	-	-
40	+ 0.23	+ 0.17	+ 0.10 (2)	+ 0.04 (10)	− 0.02 (2)	− 0.09 (1)	-	-
50	+ 0.21	+ 0.14	+ 0.07 (5)	± 0.00 (101)	− 0.07 (72)	− 0.14 (6)	− 0.20	-
60	+ 0.20	+ 0.13	+ 0.05 (9)	− 0.02 (128)	− 0.09 (187)	− 0.16 (41)	− 0.23 (2)	− 0.30
70	-	+ 0.13	+ 0.05	− 0.03 (57)	− 0.10 (134)	− 0.18 (36)	− 0.26 (13)	− 0.33
80	-	+ 0.14	+ 0.06	− 0.03 (26)	− 0.12 (63)	− 0.20 (37)	− 0.28 (6)	− 0.36 (2)
90	-	+ 0.16	+ 0.07	− 0.02 (7)	− 0.11 (15)	− 0.19 (9)	− 0.28 (9)	− 0.36 (2)
100	-	-	+ 0.08	− 0.02 (2)	− 0.11 (2)	− 0.20 (3)	− 0.29	− 0.37 (3)
110	-	-	+ 0.09	− 0.01 (1)	− 0.09 (3)	− 0.18 (1)	− 0.27	− 0.38
120	-	-	-	+ 0.05	− 0.06 (1)	− 0.16 (2)	− 0.25	− 0.39
Algebraic mean	(+ 0.23)	(+ 0.17)	+ 0.07	− 0.01	− 0.09	− 0.18	− 0.27	− 0.36

Calculation of the error when Formula 2 is used for surface area determinations:

The surface area for persons of different weights and of a height of 160 ± ΔH cm, was calculated according to the formula of Du Bois & Du Bois and to the Formula 2. The deviations between the results of these calculations are listed in Table II. As expected, different deviations were found within each height class. A mean value for the deviations of each height class was calculated with the aid of the height-weight distribution for 1000 unselected subjects measured at this laboratory. As shown in Table II these means were found to be very close to 1/100 of the term ΔH in each height class, but having the opposite sign to ΔH.

The definite formula for mental arithmetic of the surface area:

From the calculations in the preceeding sections the Formula 2 may be transformed for general use (within certain limits and demands for accuracy) to:

$$A = 1 + \frac{W + \Delta H}{100} \qquad (5)$$

where A = surface area in m², W = weight in kg and ΔH = the deviation (in cm) in body height from 160 cm. Due allowance for the sign must be made here.

The use of Formula 5 in mental arithmetic is illustrated by a few examples:

Example	1	2	3
Height (cm)	147	182	182
Weight (kg)	44	73	95
ΔH	$147 - 160 = -13$	$182 - 160 = 22$	$182 - 160 = 22$
$W + \Delta H$	$44 - 13 = 31$	$73 + 22 = 95$	$95 + 22 = 117$
$1 + \frac{W + \Delta H}{100}$	$1 + 0.31 = 1.31$	$1 + 0.95 = 1.95$	$1 + 1.17 = 2.17$

ACCURACY OF FORMULA 5:

The accuracy of the simple formula given his paper has been checked by compari- s with data reported on surface area ob- ed by direct measurements. Furthermore, as been compared with the generally used mula 1 by Du Bois & Du Bois (1916) with the recently constructed diagram calculation of the surface area, given by droy & Cecchini (1954).

nparisons with reported data from direct measurements of the surface area

Sendroy & Cecchini (1954) collected all a reported in the literature where the sur- area had been measured by means of ing or coating methods or by the linear hod of Du Bois & Du Bois (1915), in cases. The appearance of the data plotted diagram (the term (H + W) against A) sed Sendroy & Cecchini to divide the erial into three different classes with re- d to the surface area:

Subjects with a measured area below 0.8 (62 cases): The relation between + W) and A described a curved line hin these limits. Formula 5 gives too low ues; the error increases with decreasing face area. Formula 5 cannot be recom- nded for these small subjects.

Subjects with a measured area within 0.8—2.2 m² (188 cases): Sendroy & Cecchini found that the scatter of the points within these limits "indicated that a straight line relationship could be used to describe these data" and gave the following equation for the line

$$A = 0.0097\ (H + W) - 0.545 \qquad (6)$$

This equation is practically identical with that of the present Formula 5 which may be transformed to

$$A = 0.010\ (H + W) - 0.60 \qquad (7)$$

The maximal deviation between the surface area calculated according to Equations 6 and 7 (identical with Formula 5) is only 0.02 m² within the given range.

Subjects with a measured area above 2.2 m² (2 cases): The upper limit, 2.2 m², for Equation 6 was drawn by Sendroy & Cecchini on the basis of two measured subjects only. When applying Formula 5 to these two cases an error of about —0.15 m² (—5 per cent) arises. Formula 1 by Du Bois & Du Bois gave an error of —0.30 m², the diagram of Sendroy & Cecchini and error of —0.05 m².

Comparison with the Formula 1 of Du Bois & Du Bois (1916)

Calculations of the surface area according to Formulae 1 and 5 were performed during a period of 12 months on all cases remitted

Table III. *The validity of Formula 5,* $A = 1 + \frac{H + \Delta H}{100}$, *when compared with the formula of Du Bois & Du Bois and with the diagram of Sendroy & Cecchini*

Range of percentage deviation between the calculations (without regard to sign)	Comparison with Du Bois & Du Bois				Comparison with Sendroy & Cecchini			
	Material A		Material A + B		Material A		Material C	
	Number of cases	Accum. percentage	Number of cases	Accum. percentage	Number of cases	Accum. percentage	Total Number of measurements	2.5—4.2 m² Number of measurements
0.0—0.5	490	49.0	1982	98.46	240	24.0	2	1
0.6—1.5	346	83.6			203	44.3	1	0
1.6—2.5	134	97.0			296	73.9	8	4
2.6—3.5	19	98.9			159	89.8	2	2
3.6—4.5	8	99.7	21	99.50	83	98.1	3	1
4.6—5.5	2	99.9	9	99.95	15	99.6	4	1
above 5.5	1	-	1	-	4	-	2	1
Total	1000		2013		1000		22	10
Mean percentage deviation without regard to sign		—0.84	-			1.78	3.1	3.1
Algebraic percentage mean		—0.13	-			+ 0.65	+ 3.0	+ 3.1

to this laboratory for determination of the basal metabolism, a total of 2013 different subjects (Table III).

The first 1000 subjects (Material A — height—weight distribution see Table II) were collected more thoroughly, the deviation between the two calculations being recorded in each case. The results, listed in Table III, show that in 49 per cent of the total material Formula 5 gives figures for the surface area which coincide with those in Formula 1. In 97.0 per cent the deviation between the two calculations is less than ± 2.6 per cent. The algebraic mean deviation is —0.13 per cent, which excludes a systematic error in Formula 5.

In the subsequent 1013 subjects (Material B) only cases were recorded showing a deviation between the two calculations above ± 3.5 per cent, a total of only 20 cases. In one single case of the total 2013, the deviation was above 5.5 per cent.

Comparisons with the diagram of Sendroy & Cecchini (1954)

Material A was also subjected to determinations of the surface area using the recently constructed diagram of Sendroy & Cecchini.

In 24 per cent of the material the surface area calculated from Formula 5 coincides with those found in their diagram, in 89.

cent the deviation was less than ± 3.6 cent, in only 4 cases was the percentage iation above 5.5 (but below 7.5 per cent). e algebraic mean deviation was + 0.65 cent.

Due to the lack of a sufficient amount of jects of a large stature in material A, omparison was made with the 17 large ividuals, collected and calculated by Send- & Cecchini (Table III, Material C). The ebraic percentage mean deviation was 3.0, thus somewhat greater than in the vious materials and showed a small bias. two out of the 22 measurements reported deviations were above + 5.5 per cent ut below 7.5 per cent).

DISCUSSION

Despite the fact that the formula for cal- ation of the human body surface area pre- ted in this paper is very simple, it offers accuracy quite sufficient for clinical pur- ses. Only for subjects of a small stature elow 0.8 m²) does the formula give in- bitably incorrect values.

In the foregoing section it was shown that rmula 5 gives nearly the same values for surface area as those found from direct asurements on subjects within the limits —2.2 m². However, Sendroy & Cecchini 954) pointed out that most of the meas- d subjects had a normal height—weight ation, and for that reason they doubt the neral use of their simple Equation 6.

In fact, Formula 5, even for subjects of abnormal height—weight relation, seems valid as the generally accepted formula Du Bois & Du Bois. The large clinical terial studied certainly includes several h subjects, as it consists of people with a presumed pathological basal metabolism. Nevertheless, only one single case out of the 2013 cases showed a calculated area which deviated more than 5.5 per cent from that found from Formula 1 of Du Bois & Du Bois. In only 30 subsequent cases was the deviation between 3.5—5.5 per cent, which is of no practical importance in clinical work. Moreover, Formula 1 usually gives slightly higher values for the surface area than those found from direct measurements within the limits 1.30—1.80 m². In that range Formula 5 usually gives lower values than Formula 1. Above 1.80 m² Formula 1 gives a lower value than that measured, whereas Formula 5 usually gives higher values than Formula 1. Thus, the deviations found between the calculations according to Formulae 1 and 5 seem to be in favor of Formula 5 in most instances.

Formula 5 also seems to give values sufficiently comparable with those obtained from the diagram constructed by Sendroy & Cecchini (1954). They regard their diagram to be more valid than the formula of Du Bois & Du Bois, especially for subjects with abnormal height-weight relations, as it was initially based also on the shape factor W/H (for details, *loc. cit.*). Nevertheless, only 4 out of the 1000 cases calculated according to the diagram and Formula 5 showed a deviation greater than 5.5 per cent.

The agreement between Formula 5 and the diagram of Sendroy & Cecchini for subjects of a large stature (Material C) is nearly as good. One of the subjects had been recorded on five different occasions during his period of growth and affords an example of the validity of Formula 5 during changes of the shape factor W/H in the range of large surface areas. His different data were

(in kg/cm) : 81/183 — 174/252 — 197/259 — 225/264 — 223/268. The deviations between the surface areas calculated according to Formula 5 and to the diagram of Sendroy & Cecchini are + 2.0, + 4.3, + 3.4, + 2.1 and + 1.9 per cent respectively.

It may thus be concluded that the simple Formula 5 has a high validity for calculations of the surface area for subjects larger than 0.8 m². This is surprising as it must mean that above this limit an increase in height of 1 cm affects the surface area to the same degree as an increase in weight of 1 kg. From a purely scientific point of view this is certainly not true, as pointed out by Sendroy & Cecchini. Formula 5 should not be used when an exact value for the surface area is desirable. In such instances direct measurements are perhaps the only method of choice. The pecularities in the body shape of the actual human subject can never be recorded by a correction factor included in any formula. The future will show whether or not the diagram of Sendroy & Cecchini is an acceptable substitute for that very complicated operation. The formula of Du Bois & Du Bois in that respect does not seem to afford any advantages over Formula 5.

In the clinic we have to accept a formula. Most clinicians prefer a nomogram or chart based on such a formula. Then a further error is introduced, the magnitude of which depends on the quality and size of the chart and ruler in use. From that point of view a formula which allows mental arithmetic may even be superior in addition to its simplicity. Moreover the data on which the calculations are based — the figures for height and weight — depend on the status of the patient during the measurements and on the quality of the scales and the measuring tape. It seems injudicious to demand an exact formula in a calculation from inexact figures.

Finally, it has been mentioned that Formula 5 is valid only above 0.8 m². In the range 0.8—1.0 m² however, the deviation from Du Bois & Du Bois' formula as well as from the diagram of Sendroy & Cecchini is comparatively large. For that reason Formula 5 is recommended only for subjects with a calculated area larger than 1.0 m². If, for a small subject, the total of height and weight is smaller than 160 the diagram of Sendroy & Cecchini or the formula of Boyd (1935) should be used, viz.:

$$A = 3.207 \times H^{0.3} \times W^{0.7285 - 0.0188 \log W} \quad (8)$$

(where A, H and W are in terms of cm², cm and gm, respectively).

SUMMARY

1. A simple formula suitable for the mental arithmetic of the human body surface area is presented (Formula 5).

2. The formula gives values for the surface area which closely agree with those found by direct measurements on individuals above 0.8 m².

3. The formula gives values which extremely seldom deviate more than ± 5 per cent from those found with the generally accepted formula of Du Bois & Du Bois or the more modern diagram of Sendroy & Cecchini.

4. The formula is recommended for clinical purposes and only for subjects with a calculated surface area larger than 1.0 m².

REFERENCES

d, E.: The growth of the surface area of the human body. Minneapolis: Univ. Minnesota Press, 1935.

Bois, D. & Du Bois, E. F.: A formula to estimate the approximate surface area if height and weight be known. Arch. Int. Med. *17,* 863, 1916.

Du Bois, D. & Du Bois, E. F.: The measurement of the surface area of man. Arch. Int. Med. *15,* 868, 1915.

Sendroy, J., Jr. & Cecchini, L. P.: Determination of human body surface area from height and weight. J. Appl. Physiol. *7,* 1, 1954.

Scandinav. J. Clin. & Lab. Investigation 290—296, 10, 1958.

MICROMETHOD FOR THE MEASUREMENT OF COLLOID OSMOTIC PRESSURE THROUGH HYDROSTATIC COMPENSATION

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The technical difficulties involved in the measurement of colloid osmotic pressure have limited the number of clinical investigations. There has been no method suitable for serial studies. Existing methods are based on the compensation of colloid osmotic "suction" of water by a counterforce of known magnitude.

In the method reported on in the present article osmotic "suction" is compensated by hydrostatic pressure which is easy to adjust. When the colloid solution "sucks" water towards itself through the semipermeable membrane, the meniscus moves in the osmometer capillary tube. This is observed with the aid of a microscope and an endeavour is made to establish equilibrium through hydrostatic counterpressure to make the fluid level stationary so as to prevent any flow of the fluid through the membrane.

The method has proved particularly serviceable for the comparison of two colloid solutions. The osmotic differences on the opposing sides of the semipermeable membrane are not very great with this set-up and it is easy to establish equilibrium as the hydrostatic force can be made either positive or negative. The counterpressure principle introduced by Tammann (1898) and employed subsequently in several osmometers is applied in this method. Autio's method o determination (1955) is applied to the exten that the colloid solutions on both sides of th membrane are used.

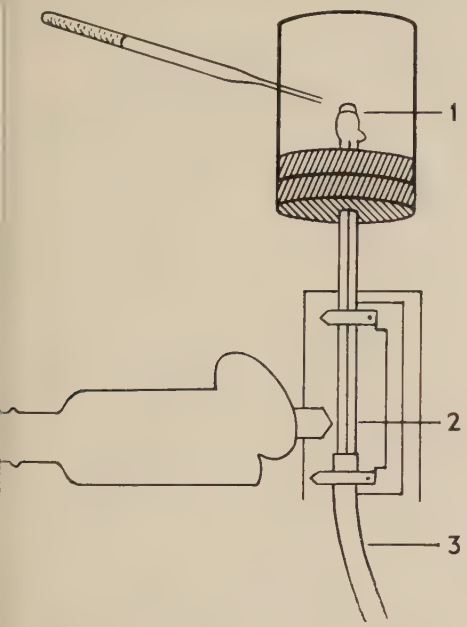
METHODS

Equipment

The equipment employed is illustrated diagram matically in Fig. 1 and 2.

The *osmometer* used, of the type introduced b Holm-Jensen (1949), consists of an 18 cm lon capillary tube of glass. One end widens out to forn an osmometer chamber with a capacity of 0 3 c The open lower end is sealed with a nitrocellulos membrane. In the method reported on the osmo meter is used with the chamber and membran turned upwards. If at least 4—5 osmometers ar available it will not be necessary to rely on jus one or two serviceable membranes.

The focal point of the osmometer is *semiper meable nitrocellulose membrane* such as Holm Jensen (1949) used except that it is a little thinner The osmometer is dipped into the membrane solu tion three times instead of four, thus increasin permeability. After the fresh membranes have drie overnight in a refrigerator they are immersed i water and the permeability to water is measure prior to use by determining the so-called minut number, *i. e.* by examining the velocity at whic water filters through the membrane under a give pressure in a given time. This requires a pressur bottle furnished with a manometer tube the struc ture and use of which are explained in the publica tion by Holm-Jensen (1949). In his method th



. 1. Osmometer attached to the microscope. Membrane and the drop of fluid outside. 2. Fluid d in the osmometer capillary tube. 3. Rubber ube connecting the osmometer to the known pressure.

est minute number of serviceable membranes is 0. In the method introduced in the present ar- the pressures on the membrane are so small it is possible to use membranes which are more meable and so expedite measuring. Membranes n a minute number of 50—70 proved the most able. No passage of the colloids *via* the mem- ne to the wrong side has yet been observed in e membranes, a danger attaching to the use of ly permeable membranes.

The *osmometric chamber* and a part of its in- orated capillary are enclosed in a spacious er, the air space of which acts as a thermostat. beaker is 7.5 cm in diameter, 9 cm deep. Two n diameter holes are bored in its walls, opposite another, 3.5 cm from the bottom. The samples he membranes are changed during the measur- process through these apertures and they are used for washing the membrane by means of llary pipettes. The apertures are sealed with ad- ve plaster.

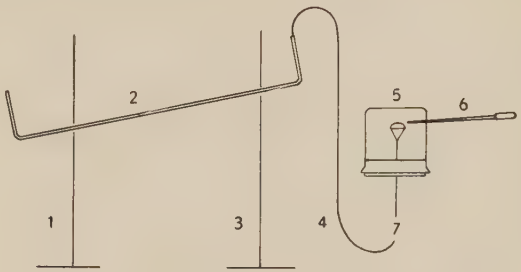


Fig. 2. A diagram of the equipment used: 1 stand on which the graduated scale is drawn — 2 hydrostatic glass tube containing water — 3 stand to hold the glass tube — 4 rubber tube connecting the glass tube to the osmometer capillary — 5 beaker with the osmometer inserted — 6 pipette, pushed through the hole in the beaker and into the drop on the membrane — 7 microscope on which the osmometer capillary is mounted and in the middle of which is the microscope tube.

The mouth of the beaker is sealed with a cork which has a hole to take the osmometer capillary and a circular drain is hollowed out on its inner surface. The drain is kept filled with water during the measuring so that saturated steam pressure is maintained inside. This prevents the drop on the membrane from being distilled into the air space.

The *microscope* is adjusted to horizontal and the osmometer and beaker protecting it are fixed in position with equipment as shown in Fig. 1. The osmometer is thus positioned vertically, with the beaker bottom and membrane upwards. The magnification of the microscope is 10 ×. Observation of the movement of the fluid level in the capillary requires a micrometer scale in the ocular. (A micrometer ocular is also needed for determination of the minute numbers of the membranes.) If the microscope stand tends to move downwards automatically under the weight of the osmometer apparatus it must be steadied. It should move only when the regulating screws are turned since other movement might be mistaken for flow of the liquid level.

The glass tube which produces the hydrostatic compensation is connected tightly to the osmometer

capillary by a rubber tube. The ends of the glass tube are bent as shown in Fig. 2 and the tube is filled with water. Some surface-active substance (*e. g.* quaternary ammonium compounds) must be added to the water to make it move readily with the slightest changes in the position of the tube. The length of the glass tube is about 95 cm, diameter 1 cm. This is sufficient for the measurement of the colloid osmotic pressure of serum but if considerably greater pressures of water, over 60 cm, are to be measured the tube must be lengthened. The difference in the height of the water levels in the arms of the tube can be adjusted as desired changing the position of the tube and this known pressure affects the fluid level inside the osmometer. The tube is fixed on a stand and a *counterweight* keeps it balanced and readily movable. On the tube arms is a mark up to which the tube is filled. The tube rests lightly on another stand.

The *graduated scale* is fixed to this second stand. On it can be read the deviation in cm between the fluid levels when the tube is in different positions. The scale is drawn by measuring the difference between the levels at 1 cm intervals. A mark is made on the glass tube at the point where the tube is horizontal on the scale. There is a mark in the osmometer at the 15 cm point in the capillary and it is to this mark that the osmometer should be filled. This 15 cm hydrostatic pressure affects the meniscus in the capillary. This can be taken into consideration when drawing the scale and the 0 point can be marked 15 cm above the horizontal plane of the tube. Upwards of the 0 point the graduations are preceded with the symbol — and downwards with the symbol +. (Taking into account the colloid osmotic pressure of the liquid on the membrane, it is to be noted that the greater this pressure the lower down the scale the hydrostatic glass tube must be moved, *i. e.* in the + direction, as compensation.)

Capillary pipettes, with a long point, are required to change the drop on the membrane.

Colloid osmotic pressure measurement

The method described has been used experimentally to measure the colloid osmotic pressure of dextran, albumin and serum. There is probably nothing in principle to prevent the similar measurement of other colloid solutions. If several samples are to be examined it helps measuring accuracy and speed of work to select a sample with which the others can be compared. The osmotic pressure of this comparative sample can then be measured against an electrolyte solution if necessary.

Preliminary measures

Care must be taken to ensure that the water levels in the arms of the hydrostatic tube reach up to their marks when the tube is horizontal, *i. e.* when the mark on the horizontal part of the tube coincides with —15 on the scale. If several samples are to be examined the one chosen as the "control sample" should be the one for which the solution is available in the greatest quantity, 0.5 cc or more. Smaller quantities are sufficient for the other samples. If the sample volume is less than 1 cc the osmometer is filled by pipetting the fluid *via* the side tube after the inside of the osmometer chamber has first been rinsed with a few drops of the solution under examination and the washing solution has been removed by suction *via* the capillary. If a more generous quantity of the sample is available the quickest way of filling the osmometer is to immerse it in the solution held in a sufficiently large test tube and to suck the solution from the end of the capillary tube through the side tube into the osmometer chamber. By tilting and applying suction carefully through the capillary the liquid level should be adjusted as closely as possible to the mark made at the 15 cm point. Surface tension is lowered in the capillary by moistening the liquid with a horsehair dipped in octyl alcohol. This measure may also be used if air bubbles tend to form in the osmometer chamber. The side tube is sealed with an air-tight rubber cap (*e. g.* a severed cystoscope stopper). The osmometer capillary is pushed through the stopper of the beaker and connected by means of a tight rubber tube to the "hydrostatic" glass tube. Before this is done, the tube must be lowered below the horizontal plane until the water levels in the arms of the hydrostatic tube are even when the rubber tube is tightly in its place at the end of the capillary. With this done the osmometer is fixed in position on the micro

e so that the spring plate of the microscope on the rubber tube encasing the end of the lary. The capillary is fixed in position *e.g.* with ip of adhesive tape close to the upper edge of tand. The fluid level in the capillary is adjusted e height of the objective.

he membrane is washed with the same solution is contained in the osmometer, dried, and the solution as is inside is pipetted onto the mem-e so that the drop covers the membrane as a e. The colloid concentration at this phase is equently the same on both sides of the mem-e. The drain in the stopper is filled with water the beaker placed in position. The edges of the per may be smeared with vaseline. The gap ining between it and the bill of the beaker ents overpressure from originating inside.

efore the measuring commences the 0 point is rmined, *i. e. the position of the hydrostatic tube n there is the same solution on both sides of the brane.* If the fluid level in the capillary is at 15 cm point it should settle motionless when the rostatic tube is at the 0 point, *i. e.* the weight of column of liquid in the capillary is compensated the colloid osmotic pressures on either side of membrane are identical. The point of equili-m is not always the theoretical, *viz.* the 0 point he scale. This is why the 0 point must always letermined prior to measuring, *i. e.* a note must nade of the reading on the scale for the hydro-ic tube when the capillary meniscus is steady and solution on both sides of the membrane is the e. It is important that this phase of the ex-ment is carried out with sufficient care and that -45 minutes be expended on it. When the move-t of the liquid in the capillary has been stopped shifting the hydrostatic tube in the necessary ction, and when the level has remained steady 5 minutes it is necessary to make sure that the ction of movement of the liquid changes when hydrostatic tube is moved from the position of librium 2 mm upwards or 2 mm downwards. state of equilibrium is noted and the change evel of the liquid in the capillary in one direc-or another from the 15 cm point is checked; value is also noted.

Measurement technique

The solution on the membrane is removed by suction with a capillary pipette. The membrane is washed several times with the solution under examination and sucking dry between washings. The solution under examination is then pipetted as a drop covering the membrane, and the apertures of the beaker are sealed with a piece of adhesive tape. There are two apertures so that when the membrane is washed and the drop changed one pipette can be held in each hand. A slight movement of the liquid level will be observed under the microscope. This is due to small temperature changes, but ceases in a couple of minutes. The glass tube is allowed to stand for about 5 minutes, whereupon it is moved in the required direction to a position where the level of the liquid remains steady. If the balance does not seem secure two positions are searched for, one of which causes an ascending the other a descending movement. The time required is 10—15 minutes and the equilibrium established is normally such that movement of the glass tube by 2 mm in either direction on the scale causes a corresponding change in the movement of the level of the liquid in the capillary. The deviation of the colloid osmotic pressure of the drop under investigation from the pressure prevailing inside can now be read from the scale. A new sample can then be placed on the membrane. It is thus possible to make some four determinations in an hour. If the 0 point deviated considerably from the theoretical 0 point, some of the serum sample in the osmometer can be reintroduced onto the membrane making sure that the 0 point coincides with its position at the outset. If this does not happen it is an indication that sufficient time has not been allowed for the establishment of equilibrium in determining the 0 point in the preliminary phase.

If the measurements are to be concluded by measuring the "absolute" colloid osmotic pressure, a drop of physiological saline solution is placed on the membrane after careful washing. The colloid solution now begins to suck water into the osmometer. This must be compensated immediately and the state of equilibrium sought.

MEASUREMENTS

750 colloid osmotic pressure measurements were carried out with the method reported.

Serum measurements

40 normal sera were examined by measuring the colloid osmotic pressure against 0.9 per cent saline solution. The values were 23.5—47 cm of water pressure. The mean, 34 cm of water pressure or 25 mm of mercury, corresponds to the values obtained by other methods; according to most investigators they range between 33 and 36 cm of water pressure (Bennhold *et al.* 1938).

64 serum samples were examined, from patients whose serum showed no protein changes. The colloid osmotic pressure was measured according to the comparative method and a parallel electrophoretic protein analysis was made of some of the samples. The intention was to find the correlation between the serum proteins and the colloid osmotic pressure, as measured by this method, in certain conditions. The results will be reported in another connection. An example is given in Fig. 3 which shows the simultaneous change in the albumin, total proteins and in the colloid osmotic pressure when 50 per cent glucose solution was injected into the test person and the blood samples were drawn in the course of 1.5 hours.

As the main emphasis was on the relative measurements, *i. e.* on eliciting colloid osmotic pressure differences, the majority of the measurements were performed by comparing two serum samples (520 measurements). A comparison of serum dilutions showed that it is possible to distinguish between two samples if the difference of their colloid osmotic pressure is 1 cm of water pressure or more. The method was also

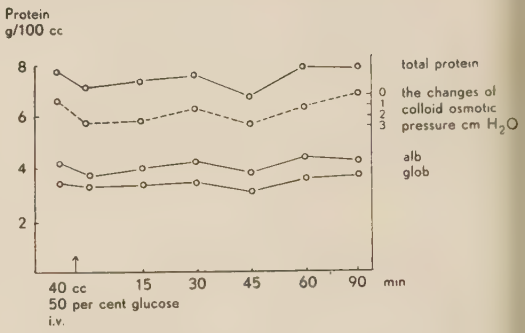
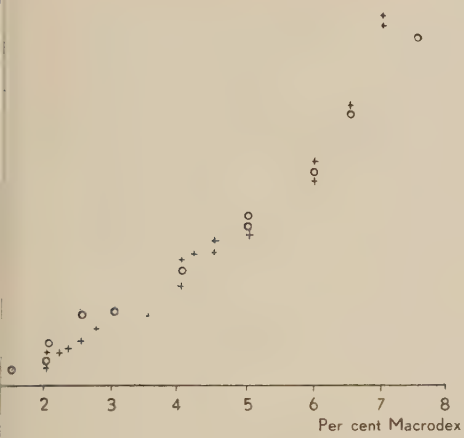


Fig. 3. Changes in serum proteins and colloid osmotic pressure after infusion of 50 per cent glucose solution.

checked by comparing samples of the "control serum" during one and the same test several times in different order. The results proved reliable provided that the 0 point had been determined carefully at the beginning of the tests.

Macrodex measurements

The colloid osmotic pressure of Macrodex solutions of different concentrations was measured against 0.9 per cent saline solution and the results were compared with values obtained by the Holm-Jensen method. As will be seen from Fig. 4, the results obtained are fairly consistent with the values given by the Holm-Jensen method. The same opinion was arrived at, however, as expressed by Neidhardt (1955), *viz.* that the Macrodex preparation used contains so many small molecules which permeate the membrane that the accurate measurement of its absolute colloid osmotic pressure is difficult. The values given were obtained in the course of 1 hour of measuring work, during which time most of Macrodex passing through the membrane had time to permeate. This is why it has not yet been possible to use Macrodex as a common "comparative colloid".



4. Colloid osmotic pressure of Macrodex solutions + the method of Holm-Jensen O present method.

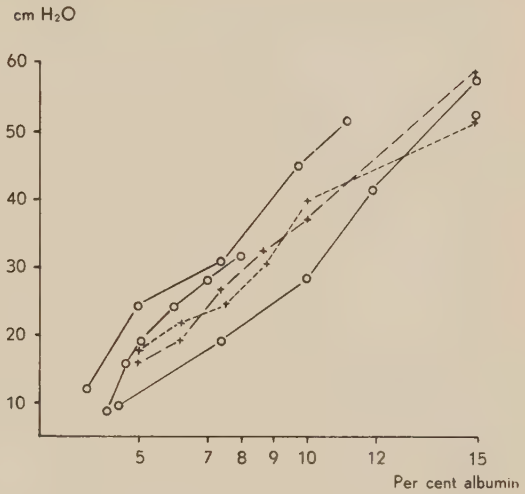


Fig. 5. Colloid osmotic pressure of different albumin solutions + - - - - + the method of Holm-Jensen, + ... + the method of Autio, o——o present method.

Albumin measurements

he colloid osmotic pressure of serum min was studied by this method and the lts were compared with the values ob- ed by the Holm-Jensen method. The tion series was prepared by dissolving min (Fraction V from bovine plasma, nour Laboratories) in 0.9 per cent saline tion. The measurements were performed n against 0.9 per cent saline solution to rmine the "absolute values" and by paring albumin solutions of different ngths. Fig. 5 gives the results. Although measurements performed with different tions are not fully comparable, it will be d that the course of the curve is essenti- the same as that of the curve obtained by Holm-Jensen method.

Measurements were also made of glucose saline solutions of different strengths to l suitable membranes and the necessary suring times. The glucose diffusion ugh the membranes employed will be re- ed on in another connection.

DISCUSSION

Biological colloid solutions usually contain particle sizes ranging from crystals to macromolecules. For osmotic pressure, information should be available on the membrane used in the measurement and on the measuring time. The result depends on these factors. With the present method the aim was to produce already at the beginning of the measurement the best possible diffusion equilibrium for the water and the particles permeating the membrane on either side, *i. e.* to produce small concentration differences. Thus the aim is to compare similar solutions. This will disclose rapidly and accurately even small deviations in the colloid osmotic pressure. A better idea is obtained of the difference between the colloid osmotic pressure of two sera by comparing them directly with one another than by comparing each of them separately with the electrolyte solution. The

most troublesome point of the method so far is the determination of the 0 point. In a state of hydrostatic equilibrium the glass tube does not always coincide with the 0 point. It may deviate a couple of cm in one direction or another. This does not happen if there is water on both sides of the membrane or saline solutions of the same strength. Hence it is a property associated with colloids and dependent also on the membrane, but just how has still to be cleared up.

Rapidity and accuracy of performance, especially in serial studies, may be regarded as the advantage of the method introduced. It would obviously be useful to have a reliable "standard colloid" against which all the measurements could be compared. This would increase the serviceableness of the method because the "absolute" values of the colloid osmotic pressure would be more comparable. There is nothing in principle to prevent measurement of "absolute" colloid pressure by the method introduced, *e. g.* against saline solution. The results given by the serum, Macrodex and albumin measurements are similar to those obtainable with other methods. Except for the microscope, the equipment needed is relatively simple and cheap.

SUMMARY

A method for the measuring of colloid osmotic pressure is reported. In the method, colloid osmotic suction is compensated by an easily adjustable hydrostatic counterpressure. The compensation required is discovered by observing microscopically the flow of the liquid in the osmometer capillary. Th method has proved very suitable for th direct comparison of colloid solutions whe there is colloid on both sides of the mem brane and the osmotic differences on eithe side of the membrane are small. The quan tity of solution needed for the measuremen is about 0.5 cc. If several samples are to b examined the first measurement takes abou 1 hour, the following some 15 minutes. Th accuracy is about ±0.5 cm of water pressur A total of 750 serum, Macrodex and albumi measurements were carried out. The mea of the colloid osmotic pressure of 40 norma sera was 33 cm of water pressure, the sam as the result achieved by most investigator using other methods. Macrodex and albumi measurements gave essentially the same re sults as the Holm-Jensen method. Specia attention is drawn to the necessity of em phasising the decisive significance of th membrane used and the measuring tim when "absolute" colloid osmotic pressur are in question.

REFERENCES

Autio, L.: Studies on the determination of collo osmotic pressure. Ann. Med. Exper. et Bio Fenniae 1955, suppl. 5.

Bennhold, H., Kylin, E. & Rusznyak, S.: Die E weisskörper des Blutplasmas. Leipzig 1938.

Holm-Jensen, I.: A method for rapid determinati of colloid osmotic pressure. Scandinav. J. Cli & Lab. Investigation *1*, 70, 1949.

Neidhardt, F.: Undersøgelser over dextrans in virkning paa serums kolloidosmotiske tryk h kaniner. Universitetsforlaget i Aarhus 1955.

Tammann, G.: Zur Messung Osmotischer Druck Ztschr. f. physik. Chem. *9*, 97, 1892.

ndinav. J. Clin. & Lab. Investigation 297—302, 10, 1958.

PREPARATION OF CRYSTALLINE PORPHYRIN ESTERS FROM PORPHYRIA URINES AND NORMAL HUMAN URINE

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While preparation of porphyrins from nes with high porphyrin content is rela- ly simple, it is difficult from urines with er porphyrin concentration to which be- g most porphyria urines. Thanks to access unlimited amounts of bovine porphyria ne the author has been able to work out nethod for preparation of porphyrins in e condition from less concentrated por- yria urines, and the technic has recently n published in detail (With, 1958). In the sent paper the use of this method, origi- ly worked out for large scale preparation h 10—20 l bovine porphyria urine, on)—2000 ml portions of human porphyria ne as well as on 20 litre portions of nor- l human urine, is described. For all the porphyria urines studied the Walden- öm esters were isolated in crystalline con- ion, and from the normal urine both uro- l coproporphyrin were prepared in cry- lline condition.

METHOD

Here only a brief summary is given, for details reader is referred to With (1958).

The urine is acidified to pH ca. 2 with HCl and ked through a 2—5 mm thick layer of talc. Then porphyrin is eluted with HCl-acetone (1/9, v/v) subsequently precipitated by addition of about ml 25 per cent NH_3 per litre of eluate, cautiously ed in small portions. The precipitate is collected on a filter, dried, esterified with H_2SO_4/methanol (1/20, v/v), extracted with $CHCl_3$, washed, dried and evaporated. Then follows purification chromatography on MgO from $CHCl_3$ (pharmacopoica quality) and next chromatography on Al_2O_3 (grade IV) from benzene/$CHCl_3$(9/1, v/v) to separate uro- and coproporphyrins. The crystallization was performed on a boiling water bath from $CHCl_3$/-methanol (uroporphyrin) or $CHCl_3$/petrol ether (coproporphyrin). All steps of the procedure were followed by watching the fluorescence with a lamp standing on the bench (With, 1957). With normal urine the red fluorescence first appeared during the chromatographic procedures because the impurities quench the fluorescence.

The methods used for analysis of the purified preparations are all described in detail in a previous paper, (With, 1958).

The analytical methods used for urine were for porphobilinogen (PBG) that of Rimington, Krol & Tooth (1956), for uroporphyrin that of Dresel, Rimington & Tooth (1956), for coproporphyrin that of Schwartz, Zieve & Watson (1951).

MATERIAL

The normal urine employed consisted of mixed portions from selected patients from the medical wards of Svendborg County Hospital, *i. e.,* cases with various neuroses, non-infectious diseases of the locomotor system, and uncomplicated peptic ulcer. Two preparations each from ca. 20 l urine were performed.

The so-called Waldenström esters — *i. e.,* the uroporphyrin ester fractions from patients with acute and chronic cutaneous porphyria — were isolated from urines from four patients with acute

porphyria and two cases of chronic porphyria of the *cutanea tarda* type, 300—2,000 ml being employed in each case.

Case 1: E. S. woman aged 27, admitted to the County Hospital of Terndrup, Jutland, with a severe, fatal attack of acute porphyria. The urine from which the Waldenström ester was isolated contained porphobilinogen (PBG) 13.7 mg per 100 ml, coproporphyrin (CP) 0.169 and uroporphyrin (UP) 4.40 mg per 100 ml.

Case 2: T. T., woman aged 33, admitted to the County Hospital, Bræstrup, Jutland, with an attack of acute porphyria with abdominal and nervous symptoms. The urine from which the Waldenström ester was isolated contained PBG 26 mg per 100 ml, CP 0.123 and UP 0.610 mg per 100 ml.

Case 3: K. P., woman aged 34, admitted to the County Hospital, Varde, Jutland, with an attack of acute porphyria with abdominal symptoms. The urine from which the Waldenström ester was isolated showed PBG 19.6 mg per 100 ml, CP 0.075 and UP 0.150 mg per 100 ml.

Case 4: P. J., man aged 25, admitted to the Medical Department N., Copenhagen County Hospital, Gentofte, with acute porphyria of the nervous type with tetraplegia. The urine from which the Waldenström ester was isolated showed PBG 29.4 mg per 100 ml, CP 0.268 and UP 1.00 mg per 100 ml.

Case 5: A. H., man aged 46, admitted to the Dermatological Department of the Finsen Institute, Copenhagen (Pt. no. 332113), suffering from a light dermatosis of the face and hands accompanied by hyperpigmentation. He denied any addiction to alcohol. The urine from which the Waldenström ester was isolated showed PBG 0.29 mg per 100 ml, CP 0.086 and UP 2.60 mg per 100 ml.

Case 6: S. R., man aged 41, admitted to the Dermatological Department of the Finsen Institute, Copenhagen (Pt. no. 323468), suffering from an atypical dermatosis partly resembling discoid *lupus erythematosus* and partly multiform erythema. He admitted addiction to alcohol. The urine from which the Waldenström ester was isolated showed no PBG, CP 0.065 and UP 0.352 mg per 100 ml.

RESULTS

The technique was found to work well both with normal human urine and porphyria urines, but during the work with the former Wood's light was useless as a guide during the first parts of the procedure because the red fluorescence of the porphyrins here was completely quenched. All the urines studied exhibited large amounts of brown non-porphyrin pigment in the raw ester, and during the chromatographic procedures this formed numerous brownish zones above the porphyrin zones, a feature which became especially well established on chromatography on Al_2O_3 of esters which had been partly liberated from non-porphyrin material on previous purification chromatography on MgO.

During the chromatography of the esters from normal urine on Al_2O_3 it was clearly visible in Wood's light that they consisted of a larger fraction of coproporphyrin and a smaller one of uroporphyrin, but nevertheless it was very difficult to obtain the coproporphyrin in crystalline condition while it was easy to crystallize the uroporphyrin from the chromatographically purified urofraction using hot $CHCl_3$/methanol. The uroporphyrin crystals (Fig. 1) were numerous enough to be subjected to washing with methanol by centrifugation and showed m.p. 287—290° C corresponding to nearly pure uroporphyrin I. No coproporphyrin crystals were obtained by attempts at crystallization from hot $CHCl_3$/methanol from the first portion of normal human urine studied; here crystals were first formed on complete evaporation of the solvent, and they were consequently contaminated with greasy material (Fig. 2) and thus not fit for accurate determination of the m.p.

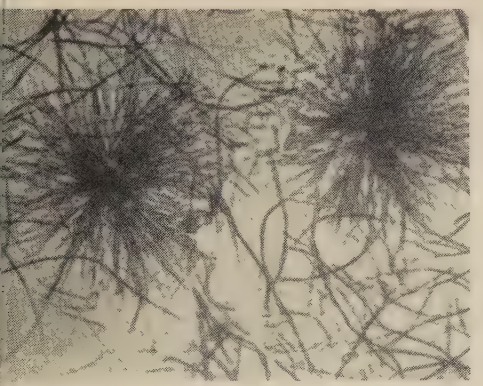

Fig. 1.

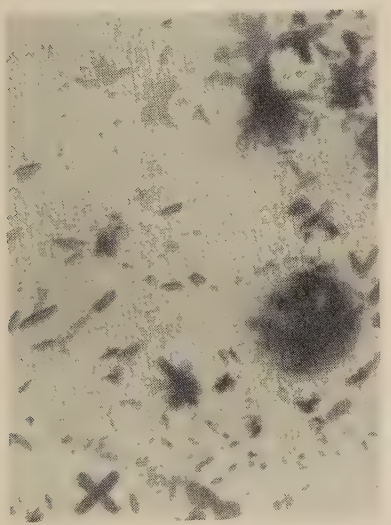

Fig. 2.

was, however, clearly visible that molli-
ation took place at about 160° C and com-
te liquefaction at about 190° C although
re-solidification was obtained because of
ich the characteristic double melting point
coproporphyrin III could not be ascer-
ned. The low melting point found is, how-
r, in itself suggestive of a high content
coproporphyrin III. In the case of the
ond portion of normal human urine a few

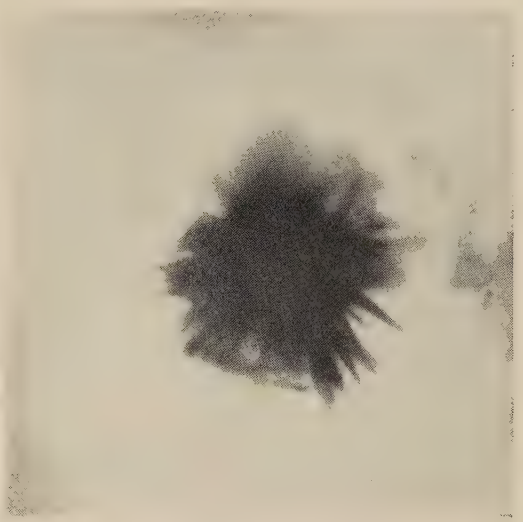

Fig. 3.

coproporphyrin crystals formed on freezing the $CHCl_3$/methanol solution before it was completely evaporated, and they were collected on a paper filter and washed with methanol. These crystals formed the rosettes of prisms characteristic of coproporphyrin III (Fig. 3), but did not allow of melting point determination because they were impossible to separate from the paper.

In the case of the normal urines the lutidine chromatography only showed very small amounts of 3- and 7- carboxyl porphyrins in addition to copro- and uroporphyrin.

The uroporphyrin fractions from the Al_2O_3-chromatography of the porphyria urines contained in all the cases by far the larger part of the porphyrins present and showed an α-band of 625 mμ in the Hartridge reversion spectroscope. The porphyrin was easy to obtain in crystalline condition from hot $CHCl_3$/methanol, and the hydrolysed esters were all found to consist of

practically pure uroporphyrin on lutidine paper chromatography. On micro melting point determination the following m.p. were found: Case 1 255—262° C, case 2 257—260° C, case 3 246—251° C, case 4 256—259° C, case 5 245—255° C, case 6 247—253° C, the first figures designating the beginning of the melting (mollification), the second its completion (liquefaction). All these melting points are characteristic of Waldenström esters.

DISCUSSION

The large amounts of non-porphyrin pigment found both in normal and porphyria urines are worthy of note, and it is interesting that already Fischer & Zerweck (1924) found these pigments in normal urine and urine from a pronounced case of human congenital porphyria. These authors stressed that the concentration of non-porphyrin pigments was roughly parallel to the porphyrin concentration and that the non-porphyrin pigments of their porphyria urine chemically were at least very closely related to the non-porphyrin pigments of normal urine. These brown non-porphyrin pigments are most likely dipyrrole pigments, so-called fuscins (cf. With, 1954) and including urochrome B (Stich & Stärk, 1953). Further, the non-porphyrin pigment porphobilin (Waldenström & Vahlquist, 1939), which is a dipyrrole, belongs here.

It is of great interest that Watson (1956) recently has mentioned some observations of his group suggesting that these pigments cannot be hemoglobin disintegration products but are byproducts of the porphyrin synthesis. Normal subjects were fed N^{15}-glycine and the stercobilin- and mesobilifuscin-fractions were isolated from their feces. It was found that all the N^{15}-containing mesobilifuscin was found in the feces collected during the first week after the glycine feeding and none in those collected after about 120 days; for stercobilin only a minor fraction of the N^{15}-containing material was found in the former fraction, the bulk being in the later corresponding to the hemoglobin breakdown

Our most remarkable finding is that the uroporphyrin of normal urine is predominantly uroporphyrin I while the coproporphyrin is mainly coproporphyrin III. But this unexpected finding is in good agreement with the few earlier studies bearing on these questions. Thus Lockwood & Bloomfield (1954) who isolated crystalline uroporphyrin from normal urine, although with a considerably more complicated technique than that described above, also found uroporphyrin I. The evidence available on the type of coproporphyrin present in normal urine is discussed by Comfort, Moore & Weatherall (1954) who conclude that coproporphyrin III predominates; further both Brugsch (1952) and Kench, Lane & Varley (1952) isolated crystalline coproporphyrin from the urine of lead-exposed human subjects and found that coproporphyrin III dominated. The former isolated typical coproporphyrin III in crystalline condition from three cases of lead poisoning while the latter isolated both coproporphyrin III and I as crystalline esters from a mixed pool of 42 litres of urine from several lead-exposed workers. About 70 per cent of the coproporphyrin present was coproporphyrin III. Comfort *et al.* (1954) who studied the porphyrins present in large amounts of normal urine did not isolate the porphyrins in crystalline condition, but with lutidine chromatography they demonstrated the presence of very small amounts of porphyrin

h 5, 6, and 7 carboxyl groups. With this hod we were only able to trace porphyrins h 3 and 7 carboxyl groups and those with copro-) and 8 (uroporphyrin).

The m.p. of the Waldenström esters varied m 245°—262° corresponding to a content either 20—40 per cent or about 80 per t uroporphyrin I according to Nicholas Rimington (1953, Fig. 1). It is noteworthy t the esters isolated with the technique cribed all consisted of practically pure porphyrin. Further it is to be pointed out t the main porphyrin of the urine in our cases of chronic (*cutanea tarda*) porria was uroporphyrin, as found by Cani- & Rimington (1953) in 8 similar cases. is fact is in disagreement with the recent posal of Waldenström (1957) to call this m of porphyria "protocoproporphyria".

SUMMARY

A procedure previously developed for isoion of crystalline porphyrins from large antities of bovine porphyria urine was nd to work well with human porphyria nes as well as with large quantities of rmal human urine. Crystalline uroporyrin isolated from the latter was found be uroporphyrin I while coproporphyrin lated from the same source was mainly proporphyrin III. These findings are in od agreement with the few earlier studies aring on the same problems. The Waldenöm esters were isolated in crystalline conion from 300—2000 ml portions of urine om four cases of acute intermittent and of chronic (*cutanea tarda*) porphyria and nd to consist of practically pure uroporyrin with either 20—40 per cent or about 80 per cent uroporphyrin I. The proposal of Waldenström to call the latter type of porphyria "proto-coproporphyria" is thus misleading.

ACKNOWLEDGEMENTS

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The author wants to thank the chiefs of the hospital departments mentioned above for generous supply of urine from their patients and to express his gratitude to Mrs. M. Rubin for valuable technical assistance.

REFERENCES

Brugsch, J.: Über die Bedeutung der Isomerenuntersuchung des Harnkoproporphyrins bei Bleivergiftung. Ztschr. Inn. Med. *7*, 378, 1952.

Canivet, J. & Rimington, C.: A chromatographic study of uroporphyrin from cases of cutaneous, acute and congenital porphyria. Biochem. J. *55*, 867, 1953.

Comfort, A., Moore, H. & Weatherall, M.: Normal human urinary porphyrins. Biochem. J. *58*, 177, 1954.

Dresel, E. I. B., Rimington, C. & Tooth, B. E.: Determination of urinary uroporphyrin by a direct extraction method. Scandinav. J. Clin. & Lab. Investigation *8*, 73, 1956.

Fischer, H. & Zerweck, W.: Über den Harnfarbstoff bei normalen und pathologischen Verhältnissen und seine lichtschützende Wirkung. Zugleich einige Beiträge zur Kenntnis der Porphyrinurine. Ztschr. f. physiol. Chem. *137*, 176, 1924.

Kench, J. E., Lane, R. E. & Varley, H.: Urinary coproporphyrins in lead poisoning. Brit. J. Indust. Med. *9*, 133, 1952.

Lockwood, W. H. & Bloomfield, B.: Crystalline uroporphyrin from normal human urine. Austr. J. Exper. Biol. Med. *32*, 733, 1954.

Nicholas, R. E. H. & Rimington, C.: Studies on the "Waldenström porphyrin" of acute porphyria urines. Biochem. J. *55*, 109, 1953.

Rimington, C., Krol, S. & Tooth, B.: Detection and determination of porphobilinogen in urine. Scandinav. J. Clin. & Lab. Investigation *8,* 251, 1956.

Schwartz, S., Zieve, L. & Watson, C. J.: An improved method for the determination of urinary coproporphyrin and an evaluation of factors influencing the analysis. J. Lab. Clin. Med. *37,* 843, 1951.

Stich, W. & Stärk, G.: Chromatographische Analyse des Urochroms B. Naturwiss. *40,* 56, 1953.

Waldenström, J.: The porphyrias as inborn errors of metabolism. Am. J. Med. *22,* 758, 1957.

— & Vahlquist, B. C.: Studien über die Entstehung der roten Harnpigmente (Uroporphyrin und Porphobilin) bei der akuten Porphyrie und ihrer farblosen Vorstufe (Porphobilinogen). Hoppe-Seyl. Ztschr. *260,* 189, 1939.

Watson, C. J.: The pyrrol pigments and hemoglobin catabolism. Minnesota Med. *39,* 294, 403, 467, 1956.

With, T. K.: Biology of Bile Pigments. Monograph, A. Frost-Hansen, Copenhagen 1954.

— A portable source of Wood's light in porphyrin analysis. Scandinav. J. Clin. & Lab. Investigation *9,* 208, 1957.

— Preparation of crystalline porphyrin esters from bovine porphyria urine. Biochem. J. 68, 717, 1958.

dinav. J. Clin. & Lab. Investigation 303—307, 10, 1958.

A COMPARISON BETWEEN THE ENZYME PATTERNS (GO-T, GP-T, LD) IN SERUM AND TISSUE EXTRACTS IN CARDIAC AND HEPATIC DISEASES

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(Received for publication March 14, 1958)

nce the initial report of LaDue, Wro- ski & Karmen (1954) on the striking sient elevation of serum glutamic-oxal- ic transaminase activity (SGO-T) in e myocardial infarction, much interest been aroused in the SGO-T level as a nostic aid. Marked elevation of SGO-T vity has also been demonstrated in a ety of diseases of the liver and bile ducts. r references see: Mason & Wrobléwski, 7; Conrad, 1957.)

everal investigators have studied other m enzymes such as glutamic-pyruvic saminase (SGP-T) and lactic dehydro- ase (SLD). As the concentrations of dif- nt enzymes vary in different organs, r relative proportions in the serum give e information concerning the organ ch is affected.

f the increase in the enzyme activities in m were due only to an escape from the otic cells, it might be suspected that the portions between the different enzymes erum in cardiac and hepatic injury should bout the same as in cardiac and hepatic ue. The purpose of this investigation was udy the proportions between the enzymes erum and tissue extracts.

METHODS

Serum glutamic-oxalacetic transaminase (SGO-T) was determined by Ordell's modification (1958) of the method of Karmen (1955).

Serum glutamic-pyruvic transaminase (SGP-T) was determined by Ordell's modification (1958) of the method of Wrobléwski & LaDue (1956). The units are equivalent in Ordell's modification and in the original methods.

In the determination of serum lactic dehydrogenase (SLD) the method of Wrobléwski & LaDue (1955) was used with some modification.

Our samples were analysed on the same day that they were taken. Hemolysis must be avoided because the LD activity in the red cells was found to be about 200 times higher than in the serum.

The determination of enzyme activity in different organs was made after homogenisation in cold saline, centrifugation for 5 min. at 2000 rpm and filtration through porcelain filters.

Normal controls: Forty assays of the serum activity from forty healthy blood donors showed the following mean values which have been taken to be the normal ranges.

SGO-T	20 ± 11	units per ml	(6— 35)
SGP-T	24 ± 9	units per ml	(8— 37)
SLD	260 ± 100	units per ml	(80—400)

RESULTS

Acute myocardial infarction

In 32 cases of myocardial infarction the SGO-T, SGP-T and SLD activities were

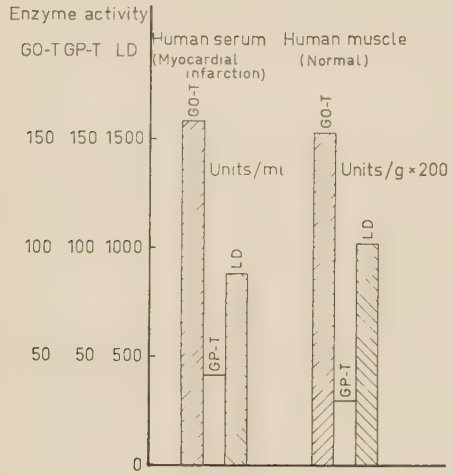


Fig. 1. The average maximal increase in the serum enzyme activities in 32 cases of myocardial infarction and the average enzyme activities in healthy striated muscle tissues from 10 human beings.

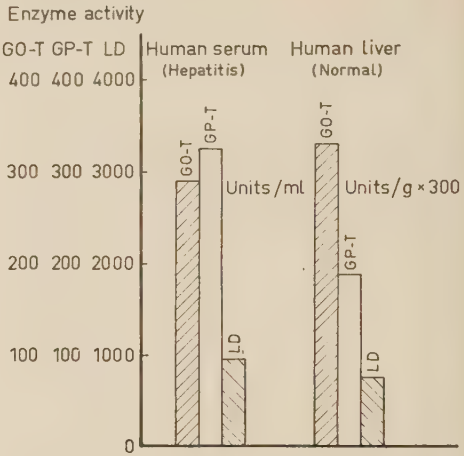


Fig. 2. The average maximal increase in the serum enzyme activities in 22 cases of epidemic hepatiti and the average enzyme activities in healthy hepati tissues from 10 human beings.

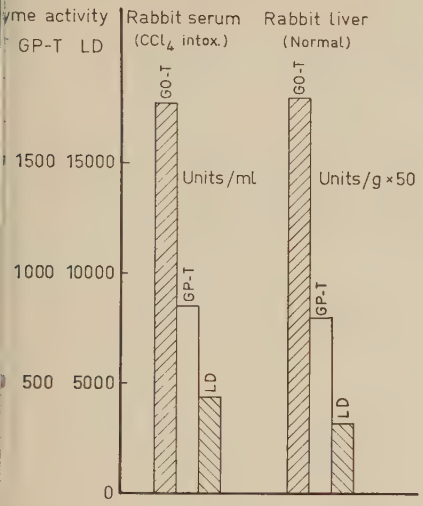
determined. In agreement with Wrobléwski & LaDue (1956), Wrobléwski, Ruegsegger & LaDue (1956) and Chinsky, Schmagranoff & Sherry (1956) we found an increase of the SGO-T and SLD activities in all cases while the SGP-T activity was normal or only slightly increased.

The enzyme activities in tissues soon decrease after death and the samples for tissue analysis must therefore be taken before or immediately after death. As fresh heart muscle is not so easy to obtain the enzyme activities have been determined in saline extracts from healthy striated muscles. The samples were taken from the abdomen in 10 patients undergoing operations. As seen from Fig. 1 we found that the proportions of the different enzymes in the muscle tissue extracts corresponded fairly well to the proportions of the enzymes in the serum in cases of myocardial infarction. The serum activities reported in this figure are th average maximal increase of each enzyme

Epidemic hepatitis

In epidemic hepatitis an increase of both the SGO-T and SGP-T is the most typica change in the enzyme pattern. In 19 out o 22 cases the increase of SGP-T was mor pronounced than that of SGO-T whereas the increase of SLD was relatively slight This is in accordance with the findings o Wrobléwski, Friend, Nydick, Ruegsegger & LaDue (1956). The proportions between th different enzymes were about the sam throughout the course of the hepatitis.

The samples for tissue analysis were take during operations from 10 patients withou hepatic diseases. Fig. 2 shows the averag maximal increase of the different enzyme of serum in 22 cases of epidemic hepatiti in relation to the enzyme activities in health



3. The average maximal increase in the serum ne activities in a rabbit receiving carbon tetra- ide and the average enzyme activities in hepatic tissues from 6 normal rabbits.

an hepatic tissue. In contrast to the find- in myocardial infarction, the propor- s of the enzymes in serum in hepatitis do correspond to the proportions in hepatic ue extracts, where the SGP-T activity is h less than the SGO-T activity.

Liver poisoning

n order to study the discrepancy in the portions between the enzymes of the m in hepatitis and the proportions in atic tissue extracts, cellular damage was duced in 8 rabbits by the instillation of on tetrachloride into the stomach by ns of a tube. The increase of the enzyme vities in the serum remained for only a days and was proportional to the dose inistered. In contrast to the findings in emic hepatitis the proportions of the erent enzymes in the serum in all the

Table I

Clinical groups	SGO-T activity	
	Normal	Increased
Chron. alcoholics without acute intoxication	17	1
Chron. alcoholics with acute intoxication	4	19

treated rabbits were about the same as in the hepatic tissue extracts from healthy rabbits. (Fig. 3.)

Chronic alcoholism

We have determined the SGO-T activity in a group of chronic alcoholics without signs of cirrhosis of the liver. The results are shown in Table I. In those patients where there was no acute intoxication on admission, an increased SGO-T activity was found in only one case, whereas in those patients who were in a state of acute intoxication on admission the majority showed an increase in SGO-T activity. In 5 patients of the latter group there were pathological flocculation reactions, which soon became normal. Otherwise there were no laboratory signs of liver damage. In all cases the increased SGO-T activity became normal again in a few days. These observations in chronic alcoholics indicate that the determination of serum transaminase activity is a very sensitive test of liver cell injury.

In 7 cases in which the chronic alcoholics had acute intoxication the SGP-T and SLD activities were also determined and the proportions between the enzymes were about the same as in normal hepatic tissue extracts and in the serum of carbon tetrachloride intoxicated rabbits.

DISCUSSION

We have tried to correlate the relative proportions of the different enzymes in serum with the ratios in tissue extracts.

Rudolph, Schaefer, Dutton & Lyons (1957) considered that the quantitative measure of GO-T activity of crude saline extracts is liable to error, because of the fact that glutamic dehydrogenase (GD) in tissue extracts is able to oxidise reduced DPN (under the same condition as in the method employed by us). We have determined the GD activities in striated muscle and hepatic tissue and found them to be very low in comparison with the GO-T activity (3—6 per cent). The determination was done in the presence of ammonia by estimating the oxidation of reduced DPN during conversion of α-ketoglutarate to glutamate. In our opinion the error resulting from the presence of GD is negligible in the determination of the GO-T activity in tissue extracts.

The relative proportions of all the estimated enzymes in striated muscle and in serum in myocardial infarction were about the same and it therefore seems reasonable to assume that the increase of the serum enzymes in this disease is primarily due to an escape of enzymes from the necrotic tissue.

The serum enzyme spectrum in epidemic hepatitis does not correspond to the proportions in hepatic tissue extracts. In hepatocellular injury due to carbon tetrachloride intoxication in rabbits, however, the proportions between the different enzymes in serum and hepatic tissue are about the same. Even in hepatic injury due to acute intoxication by alcohol the proportions seem to be the same in serum and normal hepatic tissue. As shown by Allgén (1957) there is a much higher increase of the SGO-T than the SGP-T also in patients with delirium tremens.

In view of our findings it seems justified to assume that the mechanism of the increase of the serum enzymes in epidemic hepatitis is different from that in toxic hepatocellular injury. We cannot yet explain this difference. Wroblewski, Friend, Nydick, Ruegsegger & LaDue (1956) consider that the increase in the serum enzymes during hepatitis involves in addition to the escape of enzymes from the necrotic cells, a metabolic and excretory abberation.

SUMMARY

The proportions between SGO-T, SGP-T and SLD in acute myocardial infarction were found to be about the same as the proportions of these enzymes in muscle tissue extracts.

The relative proportions between the serum enzymes in epidemic hepatitis did not correspond to those in hepatic tissue extracts.

In rabbits where hepatic damage was obtained by carbon tetrachloride intoxication and in human beings with hepatic injury due to acute intoxication by alcohol the proportions between the different serum enzymes were about the same as in hepatic tissue.

The difference in the serum enzyme patterns in hepatocellular damage due to hepatitis and intoxication are discussed.

REFERENCES

Allgén, L. G.: Personal communication.

Chinsky, M., Schmagranoff, G. L. & Sherry, S. Serum transaminase activity: observations in a large group of patients. J. Lab. & Clin. Med. *47*, 108, 1956.

ad, F. G.: Transaminase. New England. J. Med. *256,* 602, 1957.

nen, A.: A note on the spectrophotometric assay of glutamic-oxalacetic transaminase in human blood serum. J. Clin. Investigation *34,* 131, 1955.

ue, J. S., Wroblewski, F. & Karmen, A.: Serum glutamic oxaloacetic transaminase activity in human acute myocardial infarction. Science *120,* 497, 1954.

on, J. N. & Wroblewski, F.: Serum glutamic oxalacetic transaminase activity in experimental and disease states. Arch. Int. Med. *99,* 245, 1957.

ll, R.: To be published in Scandinavian J. Clin. & Lab. Investigation.

olph, L. A. & Schaefer, J. A., Dutton, R. E. & Lyons, R. H.: Serum glutamic oxalacetic transaminase in experimental tissue injury. J. Lab. & Clin. Med. *49,* 31, 1957.

Wroblewski, F., Friend, C., Nydick, I., Ruegsegger, P. & LaDue, J. S.: The mechanism and significance of alterations in serum glutamic oxalacetic and serum glutamic pyruvic transaminase in liver and heart disease. Clin. Rec. Proc. *4,* 102, 1956.

— & LaDue, J. S.: Lactic dehydrogenase activity in blood. Proc. Soc. Exper. Biol. Med. *90,* 210, 1955.

— — Serum glutamic-pyruvic transaminase in cardiac and hepatic disease. Proc. Soc. Exper. Biol. Med. *91,* 569, 1956.

— Ruegsegger, P. & LaDue, J. S.: Serum lactic dehydrogenase activity in acute transmural myocardial infaction. Science *123,* 1122, 1956.

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ON THE MECHANISM OF THE ELEVATION OF SERUM GLUTAMIC-OXALACETIC TRANSAMINASE (SGO-T) IN OBSTRUCTIVE JAUNDICE

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According to several authors the liver exerts an excretory function with regard to SGO-T. This conclusion is based on the findings of Chinsky, Schmagranoff & Sherry (1956) that fresh bile contains large amounts of this enzyme. According to these authors the elevation in obstructive jaundice may be explained by regurgitation of bile. In a study of the elevation of some DPN-linked enzymes in obstructive jaundice we also investigated the excretion of these enzymes in the bile.

METHODS

GOT was determined by Ordell's modification (1958) of the method of Karmen (1955).

The mean values and normal ranges of the SGO-T in forty blood donors were found to be 20 ± 11 units per ml (6—35 units)

RESULTS

In gall bladder bile taken during operations on 6 patients the GOT-activity showed great variations and in some cases was found to be more than 100 units per ml.

In 8 patients with fistulas in whom the gall bladder had previously been extirpated, the content of GOT in bile from the common duct was determined. In all cases the activity was low and in all cases but one did not exceed the activity of the serum (Table I).

Table I. *Results of determinations of GOT in bile from the common duct and serum collected from 8 patients.*

GOT in bile (units/ml)	SGO-T (units/ml)
5	28
8	18
12	32
12	16
19	20
26	31
28	29
42	30

In 3 cases the glutamic-pyruvic-transaminase and the lactic-dehydrogenase were also determined. The activities of these enzymes in bile from the common duct were lower than that of the serum.

DISCUSSION

Because of the low activity of the GOT in bile from the common duct we are of the opinion that the biliary elimination of the enzyme is negligible and also that the increase in the serum enzyme activity in obstructive jaundice is not due to a back flow but rather to a hepatocellular damage causing release of the enzyme. Chinsky, Schmagranoff & Sherry (1956) determined the

vity in bile taken during laparatomies in patients. The authors do not state exactly n where the bile was obtained, but it has a assumed that it was taken from the gall der. In our investigation the determina- s were made in bile from the common , and this fact might explain the discre- cy between our findings and those of nsky *et al.*

REFERENCES

Chinsky, M., Schmagranoff, G. L. & Sherry, S.: Serum transaminase activity: observations in a large group of patients. J. Lab. & Clin. Med. *47,* 108, 1956.

Karmen, A.: A note on the spectrophotometric assay of glutamic-oxalacetic transaminase in human blood serum. J. Clin. Investigation *34,* 131, 1955.

Ordell, R.: To be published in Scandinavian J. Clin. & Lab. Investigation.

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FIBRINOLYTIC ACTIVITY IN SOME HUMAN BODY FLUIDS

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Fibrinolytic systems of different types have been demonstrated in body fluids (Astrup, 1956 a, b). These systems differ especially in their content of inhibitory components. In continuation of previous investigations from this laboratory the present paper describes the fibrinolytic systems in ascitic fluid, synovial fluid, cerebrospinal fluid, sweat and some other human body fluids.

MATERIALS AND METHODS

Body fluids were collected from human beings and investigated immediately or stored for later use at —20° C.

The fibrinolytic activity was estimated by the fibrin plate method (Astrup & Müllertz, 1952). Since bovine fibrin contains large amounts of plasminogen the activities recorded could be caused either by fibrinolytic enzymes or by activators of plasminogen, but heating the fibrin plates for 35 minutes at 85° C destroys the plasminogen in the substrate (Lassen, 1952). The protease activity estimated on heated fibrin plates is therefore a measure of the content of the fibrinolytic enzymes only.

The stock solution of streptokinase (Varidase, kindly supplied by the Lederle Laboratories) containing 1 mg/ml (in saline) was diluted 1:25 in barbital buffer before use.

The stock solution of pure crystalline trypsin (25.4 Anson units/g, kindly supplied by the Novo Laboratories, Copenhagen) contained 0.25 mg/ml (in 0.05-N HCl) and was diluted 2:15 in the barbital buffer before use.

Bovine plasminogen was prepared as described by Astrup & Sterndorff (1953). The stock solution contained 20 mg/ml.

A highly purified preparation of the human urine activator (Ploug & Kjeldgård, 1956) was kindly placed at our disposal by Løvens kemiske fabrik, Copenhagen. The concentration used was 2 mg/ml.

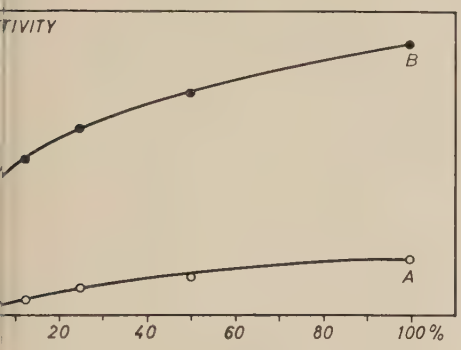
The diethyl barbiturate buffer (Michaelis, 0.05 M, pH 7.75) contained 0.1 M NaCl. Total $\mu = 0.15$.

RESULTS

Ascitic fluid:

Samples were collected from patients suffering from liver and heart diseases. Ascitic fluid from cancer patients and fluids contaminated with blood were not investigated.

In 8 of 10 samples from different persons small spontaneous activities were found on normal fibrin plates. Addition of diluted streptokinase solution produced a large increase in activity in all cases (Fig. 1). Streptokinase alone had no or only an extremely small lytic effect on the plates. When the samples (without streptokinase) were applied to heated fibrin plates a slight digestion was obtained in two cases only. These results demonstrate the presence of small amounts of an activator of plasminogen in most of the cases and a considerable amount of a precursor of a plasminogen activator in all cases. In only two cases were small amounts of a fibrinolytic enzyme recorded.



1. Activity of serial dilutions of ascitic fluid
andard fibrin plates. Ascitic fluid (1 ml) +
tal buffer (0.5 ml) (curve A). Ascitic fluid
ml) + streptokinase (0.5 ml) (curve B).
issa: Concentration of ascitic fluid in per cent
of stock solution.
nate: Activity as product of 2 diameters (mm^2)
of the lysed zones.

fter addition of streptokinase all samples
luced slight activity on the heated fibrin
cating the presence of small amounts of
minogen in the samples which was ac-
ed by the plasminogen activator formed.
n an excess of bovine plasminogen was
d to the spontaneously active samples
the solutions then applied to the heated
in plates an increase in the activity was
rded demonstrating the presence of a
minogen activator. This activity was
ner increased by addition of both plas-
ogen and streptokinase to the original
ples (Fig. 2). Minimal or no activity was
rded when streptokinase and bovine
minogen were applied alone or in mix-
to heated fibrin plates. These results
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ic fluid contains small amounts of an
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precursor of a plasminogen activator.

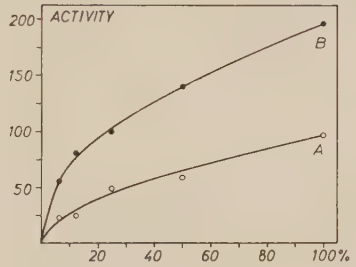

Fig. 2. Activity of serial dilutions of ascitic fluid on heated fibrin plates. Ascitic fluid (1 ml) + barbital buffer (0.5 ml). No activity. Ascitic fluid (1 ml) + bovine plasminogen (0.5 ml) (curve A). Ascitic fluid (1 ml) + bovine plasminogen + streptokinase (0.5 ml) (curve B).
Abscissa and ordinate: Same as Fig. 1.

In order to substantiate the existence of plasminogen in the ascitic fluid, equal volume of a solution of the urine activator was added and the mixture applied to heated plates. Slight digestion of the substrate was obtained by this procedure whereas no effect was obtained with the urine activator alone (Fig. 3).

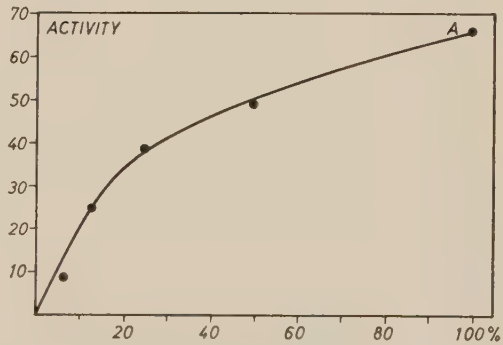

Fig. 3. Activity of serial dilutions of ascitic fluid and urine activator on heated plates. Ascitic fluid (0.5 ml) + barbital buffer (0.5 ml). No activity. Ascitic fluid (0.5 ml) + urine activator (0.5 ml) (curve A).
Abscissa and ordinate: Same as Fig. 1.

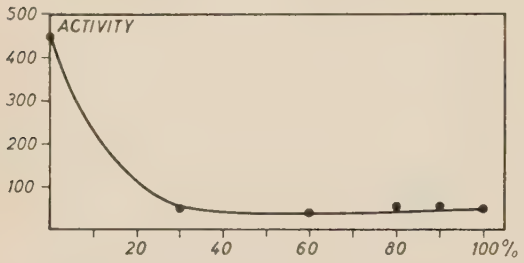

Fig. 4. Inhibition of trypsin (1 ml) by ascitic fluid in different concentrations (1 ml) on heated plates. Abscissa: Concentration of ascitic fluid in per cent of undiluted sample.
Ordinate: Same as Fig. 1.

The activity of trypsin was decreased by addition of ascitic fluid to the trypsin solution, which demonstrates the presence of inhibitory agents in this fluid (Fig. 4).

In order to characterize the plasminogen activator present in spontaneously active ascitic fluid its stability at various pH values and temperatures was investigated in the following manner: The pH was adjusted with 0.1 N HCl, 0.1 N NaOH or various buffers (citrate, phosphate and borate) after which the samples were heated for 30 minutes at 37° C, 50° C, 70° C and 100° C. They were then cooled to 0° in ice water, neutralized, and immediately applied to standard plates in order to estimate the remaining activity (Fig. 5). The activator was destroyed at 70° C and 100° C. At 37° C and 50° C it was destroyed at acid reaction but was stable at more alkaline pH.

The activator produced by addition of streptokinase showed similar stabilities at the various temperatures and pH values (Fig. 6 A). These results indicate that the activator spontaneously present and the activator produced by the addition of streptokinase are probably analogous.

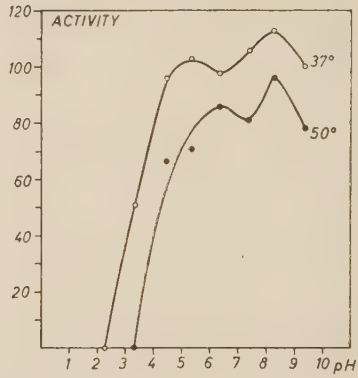

Fig. 5: Fibrinolytic activity of ascitic fluid o standard plates after heating for 30 minutes a 37° C and 50° C.
Abscissa: pH values of the samples during heating
Ordinate: Activity as product of 2 diameters (mm^2) of the lysed zones.

The heat stability of the inhibitor was in vestigated in the same manner except tha after neutralization the solutions were adde to equal amounts of a trypsin solution an the amount of trypsin remaining estimate on heated fibrin plates (Fig. 7 A). The in hibitor was destroyed at 70° C and 100° C At 37° C and 50° C it was destroyed at aci reaction but remained stable at more alkalin reactions.

Synovial fluid:

Eight samples of synovial fluid from pa tients suffering from noninflamed hydar throns were removed by aspiration. Th samples were investigated as described fo ascitic fluid.

No spontaneous activity was recorded fro any of the samples when applied to standar plates. The addition of streptokinase pr duced a considerable increase in activit (Table I) indicating the presence of a larg

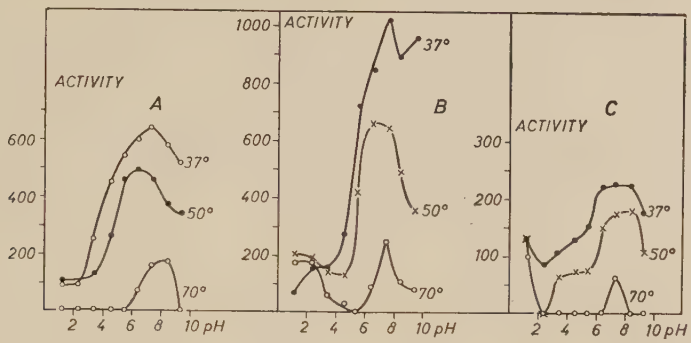

Fig. 6. Fibrinolytic activity of ascitic fluid (A), synovial fluid (B) and cerebrospinal fluid (C) on standard fibrin plates after addition of streptokinase and heating for 30 minutes at 37° C, 50° C and 70° C.
Abscissa and ordinate: Same as Fig. 5.

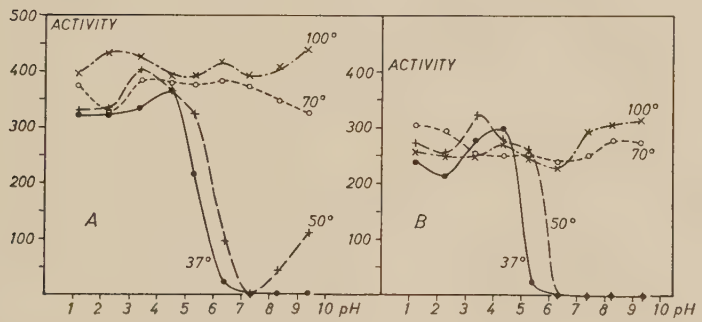

Fig. 7. Remaining activity of trypsin after addition of ascitic fluid (A) and synovial fluid (B) (heated for 30 minutes at 37° C, 50° C, 70° C and 100° C) on heated plates.
Abscissa and ordinate: Same as Fig. 5.

unt of the precursor of a plasminogen vator. The results thus obtained were tantiated by the addition of bovine plasogen and streptokinase to the samples application to heated plates. Table I vs the results of a typical experiment.

he presence of plasminogen was demoned when the urine activator was added the mixture applied to heated plates ble I). Furthermore, large amounts of trypsin inhibitor were found in the bles (Table I).

The stabilities of the activator formed by addition of streptokinase, and of the inhibitor at different temperatures and pH values were investigated as described for ascitic fluid. Prior to this investigation it was necessary to precipitate the fibrinolytic components from the synovial fluid with acetone. The procedure was as follows: 40 ml ice-cold acetone was added to 10 ml synovial fluid while stirring constantly. The solution was left in ice water for 15 minutes, after

Table I. *Investigation of the activity in ascitic fluid, synovial fluid, cerebrospinal fluid and sweat. Digested zones on standard fibrin plates and heated fibrin plates (in mm²) after applying the different mixtures and incubation for 20 hours at 37° C.*

Addition	Standard plates		Heated plates				
	Barbital buffer (0.5 ml)	Streptokinase (0.5 ml)	Barbital buffer (0.5 ml)	Plasminogen + streptokinase (0.5 ml)	Urine activator (1 ml)	Trypsin (1 ml)	Trypsin control
Sample (1 ml)							
Synovial fluid	0	694	0	174	107	0	462
Cerebrospinal fluid	0	59	0	84	0	405	429
Sweat	0	0	0	0	0	374	367
Ascitic fluid	130	630	0	195	66	50	450

which it was centrifuged. The same amount of cold acetone was then added to the precipitate and after a second treatment the precipitate was dried with ether. Solutions were prepared by redissolution in the original volume of barbital buffer.

The stability curves obtained for the activator (Fig. 6 B) and the inhibitor (Fig. 7 B) corresponded to the curves obtained with ascitic fluid. Therefore the components are probably identical.

Cerebrospinal fluid:

Six samples were obtained by spinal puncture and investigated as described above.

No spontaneous activity was found on either the standard or the heated plates, whereas the addition of streptokinase produced activity on the standard plates. Table I shows an example. Streptokinase alone had no effect on the plates. So far the cerebrospinal fluid seems to be similar to the synovial fluid and this suggestion was further substantiated by the stability curves (Fig. 6 C).

However, a difference exists between cerebrospinal fluid and certain other body fluids since no plasminogen and no inhibitory agents against trypsin were present (Table I).

Sweat:

Five samples of human sweat were obtained by heating one arm and collecting the sweat from the other by means of a rubber bag.

No activity was found on standard or heated fibrin plates either before or after the addition of streptokinase and no plasminogen or inhibitory agents against trypsin could be demonstrated (Table I).

Other body fluids:

Fluids from hydrocele, blisters and lymph (from a lymph node fistula) were collected and investigated. In these fluids no spontaneous activity was found on either standard or heated fibrin plates, but a large amount of proactivator was demonstrated by the addition of streptokinase. The inhibitory effect against trypsin was large in all cases (Table II).

Table II. *Investigation of the activity in hydrocele fluid, fluid from blisters and lymph. Digested zones on standard and heated fibrin plates (in mm²) after applying different solutions of the fluids and incubation for 20 hours at 37° C.*

	Standard plates		Heated plates		
Addition	Barbital buffer (0.5 ml)	Strepto-kinase (0.5 ml)	Barbital buffer (0.5 ml)	Trypsin (1 ml)	Trypsin control
ple (1 ml)					
rocele fluid	0	103	0	0	144
d from blisters	0	1369	0	0	515
ph	0	514	0	0	371

DISCUSSION

ecent investigations have shown the plexity of the fibrinolytic system in an blood (Müllertz & Lassen, 1953; llertz, 1956). The fibrinolytic enzyme, smin, exists in the blood as a precursor, sminogen. The transformation of plasmi- en to plasmin can be caused by different vators. Certain tissues contain an activa- of plasminogen (Astrup & Permin, 7), and a plasminogen activator is also nd in the urine (Williams, 1951; Astrup Sterndorff, 1952). It has further been wn that normal human blood contains a cursor of a plasminogen activator which be transformed into a plasminogen acti- or by streptokinase (Müllertz & Lassen, 3). Inhibitory agents against this system st in the blood.

The body fluids investigated in this and previous papers belong to three different es: Secretions, transudates and patho- cal transudates (Table III).

Secretions:

Milk (Astrup & Sterndorff, 1953), tears orm, 1955), saliva (Albrechtsen & Thay- 1955), seminal fluid (Kaulla & Shettles,

Table III. *The composition of the fibrinolytic systems in various body fluids.*

	Pro-activator	Activator	Inhibitor
Normal blood	+	0	+
Active blood	+	+	+
Milk	+	(+)	(0)
Tears	+	(+)	(0)
Saliva	+	(+)	(0)
Sweat	(0)	(0)	(0)
Seminal fluid	+	+	+
Cerebrospinal fluid	+	0	0
Amniotic fluid	+	0	+
Ascitic fluid	+	(+)	+
Synovial fluid	+	0	+
Hydrocele fluid	+	0	+
Fluid from blisters	+	0	+
Lymph from fistula	+	0	+

1953; Lundquist, Thorsteinsson & Buus, 1955; Rasmussen, Albrechtsen & Astrup, 1957) and sweat are all normal fluids secreted from glands in the organism. They can be obtained in sufficient amounts for investigation. Although certain similarities exist in these fluids with regard to the fibrinolytic system, differences are also recorded. They all contain varying amounts of activating components and are further characterized by the absence of inhibitory agents against

trypsin. The sweat is completely inactive. However, the samples investigated were obtained from persons who were stimulated in order to get a sufficient amount of fluid. Previously it has been shown that the amount of activator and proactivator in parotid saliva decreases during stimulation and a similar reaction could occur with sweat. It is therefore possible that the concentration of the activating components has been too small during the stimulation to allow determination.

Seminal fluid differs from the above-mentioned fluids by its content of large amounts of inhibitory components. The activating agents in seminal fluid correspond to those in the other secretions.

All secretions mentioned have to pass through narrow channels and the formation of fibrin clots in these channels would be harmful to the organism. The presence of a fibrinolytic system in the secretions might contribute to the resolution of such fibrin clots and could therefore be of great importance to the organism, as already suggested (Astrup & Sterndorff, 1953).

Transudates:

The cerebrospinal fluid and the amniotic fluid are normal transudates. The fibrinolytic system in amniotic fluid (Albrechtsen & Trolle, 1955) is similar to the system in normal blood since it contains proactivator and inhibitory components. However, the cerebrospinal fluid differs from the blood and from the amniotic fluid in that it contains no inhibitory components against trypsin, thus approaching the composition of the secretions. The cerebrospinal fluid has to pass through narrow channels in the brain, and occlusions here by fibrin clots could be prevented by an activation of the fibrinolytic system in the fluid. Streptokinase has been used in the treatment of fibrinous exudates in tuberculous meningitis. By lysis of intraspinal fibrin clots and adhesions, the risk of raised intracranial pressure is decreased and the effect of antibiotics considerably increased (Cathie & Macfarlane 1950; Fletcher, 1954).

Pathological transudates:

The fibrinolytic system in the pathological transudates corresponds to the system in blood. It is of special interest that most samples of ascitic fluid showed spontaneous activity. The fluid is therefore able to hamper the formation of fibrin deposits in the peritoneal cavity. Streptokinase injected into the peritoneal cavity has been used to prevent or redissolve fibrin deposits and adhesions (Wright, Smith, Rothman, Quash & Metzger, 1950). The fibrinolytic system in ascitic fluid here described may explain the effect obtained by this procedure. However, Gustavson et al. (1955) could not confirm these experiments with streptokinase and found that only plasmin prevented the formation of adhesions.

The composition of the fibrinolytic system in transudates (except the cerebrospinal fluid) and in pathological transudates apparently corresponds to the system in the blood, and the agents involved are probably identical, since both the activating and the inhibiting components are labile at acid reaction. This possibly indicates that the components are derived from the blood by a simple filtration.

The composition of the fibrinolytic system in the secretions (except the seminal fluid) differs from the system in the blood since

inhibitory components against trypsin are sent. It has been suggested as a tentative planation that the active agents in the retions are excreted by diffusion from the od plasma to the lumen of the gland, but t the glandular epithelium should be se-tively permeable to the active components th-holding the inhibitory agents.

The plasminogen activator, which is either ntaneously present or formed by addition streptokinase in the blood and in the body ids, is completely different from the plas-nogen activator present in the tissues, ce the tissue activator is stable, especially acid reaction.

The fibrinolytic system in seminal fluid fers from the system in the other secretions estigated. Recent investigations indicate t the plasminogen activator in seminal id belongs to the stable tissue activator e and not to the labile blood type. An-er difference between the seminal fluid d the other secretions is the presence of ibitory components in seminal plasma.

SUMMARY

1. The fibrinolytic systems in various man body fluids have been investigated.

2. A precursor of a plasminogen activator is found in cerebrospinal fluid, ascitic fluid, novial fluid, hydrocele fluid and fluids m blisters and lymph. Ascitic fluid showed ntaneous activity and contained a plasmi-gen activator. Sweat was apparently in-ive.

3. Trypsin inhibitors were found in ascitic id, synovial fluid, hydrocele fluid, fluid m blisters and lymph, whereas cerebro-nal fluid and sweat contained no inhibitory mponents.

4. The fibrinolytic systems in the body fluids can be classified in groups according to their composition.

ACKNOWLEDGMENT

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REFERENCES

Albrechtsen, O. K. & Thaysen, J. H.: Fibrinolytic activity in human saliva. Acta Physiol. Scandinav. *35,* 138, 1955.

Albrechtsen, O. K. & Trolle, D.: A fibrinolytic system in human amniotic fluid. Acta Haematol. *14,* 376, 1955.

Astrup, T.: Fibrinolysis in the organism. Blood *11,* 781, 1956 a.

— Biological significance of fibrinolysis. Lancet *15,* 565, 1956 b.

— & Müllertz, S.: The fibrin plate method for estimating fibrinolytic activity. Arch. Bioch. Bioph. *40,* 346, 1952.

— & Permin, P. M.: Fibrinolysis in the animal organism. Nature *159,* 681, 1947.

— & Sterndorff, I.: An activator of plasminogen in normal urine. Proc. Soc. Exper. Biol. Med. *81,* 675, 1952.

— — A fibrinolytic system in human milk. Proc. Soc. Exper. Biol. Med. *84,* 605, 1953.

Cathie, I. A. B. & Macfarlane, J. C. W.: Adjuvans to streptomycin in treating tuberculous meningitis in children. Lancet *16,* 784, 1950.

Fletcher, A. P.: Intrathecal fibrinolysis with streptokinase in tuberculous meningitis. J. Clin. Invest. *33,* 69, 1954.

Gustavsson, E., Blombäch, B., Blombäch, M. & Wullen, P.: Plasmin in the prevention of adhesions. Acta Scandinav. *109, 327,* 1955.

Kaulla, K. N. v. & Shettles, B.: Relationship between human seminal fluid and the fibrinolytic system. Proc. Soc. Exper. Biol. Med. *83,* 692, 1953.

Lassen, M.: Heat denaturation of plasminogen in the fibrin plate method. Acta Physiol. Scandinav. *27*, 371, 1952.

Lundquist, F., Thorsteinsson, T. & Buus, O.: Purification and properties of some enzymes in human seminal plasma. Biochem. J. *59*, 69, 1955.

Müllertz, S.: Mechanism of activation and effect of plasmin in blood. Acta Physiol. Scandinav. *38*, suppl. 130, 1956.

— & Lassen, M.: An activator system in blood indispensable formation of plasmin by streptokinase. Proc. Soc. Exper. Biol. Med. *82*, 264, 1953.

Ploug, J. & Kjeldgård, N. O.: Isolation of a plasminogen activator (urokinase) from urine. Arch. Biochem. Biophys. *62*, 500, 1956.

Rasmussen, J., Albrechtsen, O. K. & Astrup, T.: The fibrinolytic activity in the human prostate and in seminal fluid. 6th European Haematology Congress, Copenhagen, 1957. In press.

Storm, O.: Fibrinolytic activity in human tears. Scandinav. J. Clin. Lab. Investigation *7*, 239, 1955.

Williams, J. R. B.: The fibrinolytic activity in urine. Brit. J. Exper. Path. *32*, 530, 1951.

Wright, L. T., Smith, D. H., Quash, E. T. & Metzger, W. I.: Prevention of postoperative adhesions in rabbits with streptococcal metabolites. Proc. Soc. Exper. Biol. Med. *75*, 602, 1950.

ndinav. J. Clin. & Lab. Investigation 319—322, 10, 1958.

PAPER CHROMATOGRAPHIC SEPARATION OF THE COPROPORPHYRIN ISOMERS I AND III

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Until relatively recently almost all methods separation and purification of porphyrins re been based on the esterification of the phyrins with methyl alcohol after a preli- ary separation of the porphyrins accord- to their solubility in different solvents. ese methods were, however, much too de for the separation and identification porphyrins in trace amounts only. Only certain pathological conditions with highly reased excretion of porphyrins and/or phyrin precursors, has sufficiently strong dence been presented indicating the pre- ce of porphyrins with seven and five l groups (for references see Lemberg & gge, 1949).

The introduction of paper chromatography a method for the separation of the free phyrins according to their number of lic groups by Nicholas & Rimington 49) opened fundamentally new and revo- onary possibilities in porphyrin research, the existence of porphyrins with any nber of acidic groups from 1 to 8 in ex- a and organs under normal, pathological experimental conditions, was rapidly re- ted by a series of different workers (for erences see Rimington, 1957).

till our methods are incomplete especially n dealing with isometric porphyrins. ne paper chromatographic methods for the separation of the III and I isomers of uroporphyrin (Falk & Benson, 1953) and coproporphyrin (Chu, Green & Chu, 1951; Chu & Chu, 1957; Rappoport, Calvert, Loeffler & Gast, 1955) have been published. All these methods, however, are based on the esterification of a mixture of the isomers by methyl alcohol, purification of the esters by different means and final separation of the isomers by paper chromatography.

The relatively complex analytical procedure needed when esterification of the porphyrins is used as a means for further separation and purification of porphyrins, introduces a series of possibilities for loss of porphyrins and artefact formation. The need of better analytical methods is strongly felt by most workers in porphyrin research. In this laboratory we therefore have done very much work on new and better methods, trying to avoid esterification, and a series of methods have been published (Eriksen, 1953, 1957, 1958) making it possible to separate porphyrins completely according to the number of acid groups. However, in the case of the separation of the isomers, esterification could not be avoided.

The observation of Miss A. Benson (Falk, 1954 a, b, 1955) that small modifications in our own paper chromatographic method for the separation of porphyrins according to

their number of acidic groups (Eriksen, 1953) was very promising, making it possible to split all coproporphyrin isomers into 3 spots containing the II, III + IV, and I isomers.

The details of the methodical modifications were published in a paper by Falk, Dresel, Benson & Knight in 1956. Instead of the ascending technique used by us, they use the descending technique working in an atmosphere of the developing mixture (2—6 lutidine/water 5/3,5) and ammonia, placing troughs filled with the lutidine/water mixture and ammonia respectively in the bottom of the chromatographic chamber. The dimensions of the troughs and the amounts of the liquids had to be tested out for each chromatographic chamber. In this laboratory we have had some trouble in handling the method, we therefore tried to modify the method ourselves using the original ascending technique, and found that the original method could be used as such if only the atmosphere in the chromatographic chamber was allowed to be saturated with the developing mixture and ammonia for at least 30 minutes prior to the introduction of the paper cylinder containing the porphyrins to be analyzed. To avoid too great disturbances of the atmosphere during the introduction of the paper cylinder, the whole chromatographic set-up was turned upside down. The Petri dish, containing the developing mixture and the small beaker with ammonia, was placed in the bottom of the glass cylinder used as chromatographic chamber. These modifications not only gave a satisfactory and reproducible separation of the III and I isomers of coproporphyrin and in the same order as in the Benson modification, but also split other porphyrins with numbers of acidic groups different from coproporphyrin into more than one spot. This, however, will be dealt with in a separate communication.

We did not have to our disposal the II and IV isomers. However, as both Falk and we have obtained the separation of the III and I isomers by only slight modifications of the same method, and as the behavior of the two isomers in both methods is the same, there is no reason to assume that the II and IV isomers should behave differently in the two methods.

One big advantage of the present method is that the chromatographic set up is very simple and that no special skill in chromatography is needed.

METHOD

The chromatographic chamber consists of a glass cylinder 25 cm high and 12 cm wide, covered with a plane glass plate. In the bottom of the glass cylinder a Petri dish is placed containing the developing mixture of 2—6 lutidine/water. In the center of the dish a smaller cylinder 5 cm high and 3 cm wide 2/3 full of concentrated ammonia is placed. The atmosphere is saturated for at least 30 minutes prior to the introduction of the paper cylinder containing the porphyrins to be analyzed.

The porphyrins might be applied either in spots or strips. A convenient diameter of the spots is approximately 5 mm allowing up to 8 samples to be analyzed on one paper cylinder with at least two marker spots on the same paper.

As adsorption of porphyrins to small talc columns is used as a standard procedure in this laboratory for the final concentration of the porphyrin fractions obtained by various means, paper chromatography (Eriksen, 1953), column chromatography (Eriksen, 1957), zone electrophoresis in agar gel (Eriksen, 1958), the porphyrins are applied dissolved in a mixture of 10 per cent NH_3 and acetone 3/10, which we have found to be the strongest eluent for porphyrins adsorbed to talc, but obviously other volatile solvents might be used.

he porphyrins are applied to the paper by s of a capillary pipette of the type used in aboratory to avoid bumping in vacuum destil- h. A number of Pasteur pipettes are usually up and when a capillary pipette is needed, hin end of the Pasteur pipette is drawn out the capillary. The concentrated solution of por- in to be analyzed is placed in the wide end of pipette. The filled pipette is used as a pencil, spots or strips can be kept extremely narrow. he speed of delivery of the solution can be ated simply by cutting the capillary with a or, the nearer the wide end of the pipette the rapid the delivery. The solvent is evaporated airblast.

he type of paper used is Watman No. 1 for natography. We usually cut the paper into s 23 cm long and 19 cm wide, but other dimen- can be used. The amount of porphyrin to be zed can be varied from just visible under V light to intensely red due to the porphyrins color. When the desired amount of porphyrin been applied, the paper sheet is formed into a der and placed rapidly into the Petri dish in saturated chromatographic chamber. The cover is put into place again and the developing ure allowed to travel to the top of the paper. , however, is not necessary. Satisfactory sepa- n can be obtained even when the solvent has lled only half-way to the top. When the amount orphyrin applied to the paper is high, the dia- r of the spots or, in case of strip application, width of the zones have a tendency to increase uch that it is recommended to use the full ht of the paper to get a satisfactory separation he isomers.

sually we start the chromatography in the ing taking out the fully developed chromato- i in the morning. When working in day light autions must be taken to avoid unnecessary uction of the porphyrins by light. No special autions are needed to keep the temperature in chromatographic room constant.

he ratio of lutidine/water can be varied depend- on the porphyrins to be separated. In our ori- method we used a constant ratio of 5 parts utidine to 3 parts of water for all types of

Table I. *Rf-values of 3 COOH porphyrin and the III and I isomers of coproporphyrin chromatographed in a previously saturated chamber using 2—6 lutidine/water (5/2) and ammonia vapors as developing system.*

Porphyrin	3 COOH	Copro III[1]			Copro I[2]		
		1	2	3	1	2	3
Rf values	0.55	0.25	0.25	0.27	0.18	0.17	0.18

[1] kindly supported by Prof. C. Rimington.
[2] » » » Dr. med. T. K. With.

porphyrin. In this system the Rf-values are a linear function of the number of acidic groups in the porphyrins. The difference in the Rf-values of for instance porphyrins with 3 and 4 acidic groups is so small that it would be difficult to tell whether a new spot between these two porphyrins in the modified method had arisen from the one or the other porphyrin. When the ratio of lutidine/water is increased to 5/2 the difference in Rf-values increases so much that the origin of new spots is easily recognized (Table I).

When porphyrins with higher numbers of acidic groups are to be analyzed, an increase in the amount of water keeping the amount of lutidine constant seemes to be equally satisfactory. The possibilities of the method are not fully clarified, as we lack the necessary porphyrins. However, we hope that in a short time we will have enough of the higher porphyrins to investigate in detail the possibilities and limitations of the method.

SUMMARY

A simple and reliable method for the separation of the I and III isomers of free coproporphyrins has been described.

The method is a modification of our previous method for the separation of porphyrins according to the number of acidic groups.

REFERENCES

Chu, T. C., Green, Agnes Ann & Ju-Hva Chu, Edith: Paper chromatography of methyl esters of porphyrins. J. Biol. Chem. *190,* 643, 1951.

— & Ju-Hva Chu, Edith: Chromatographic separation of uroporphyrins I and II and the nature of Waldenström porphyrin. J. Biol. Chem. *227,* 505, 1957.

Eriksen, L.: Paper chromatography of porphyrin pigments. Scandinav. J. Clin. & Lab. Investigation *5,* 155, 1953.

— Large scale separation of porphyrin pigments on cellulose powder columns. Scandinav. J. Clin. & Lab. Investigation *9,* 97, 1957.

— Zone electrophoresis as a means of establishing the number of acidic groups in porphyrin pigments. Scandinav. J. Clin. & Lab. Investigation, *10,* 39, 1958.

Falk, J. E.: Patterns of porphyrin excretion. Biochem. Soc. Symp. *12,* 17 1954 a.

— Porphyrins. Brit. Med. Bull. *10,* 211, 1954 b.

— Haem and porphyrin formation from glycine, aminolaevulic acid and porphobilinogen. Ciba Foundation Conference, 1955, p. 63.

Falk, J. E. & Benson, A.: Separation of uro porphyrin esters I and III by paper chromatography. Biochem. J. *55,* 101, 1953.

— Dresel, E. I. B., Benson, A. & Knight, B. C. Studies on the biosynthesis of blood pigments 4. The nature of the porphyrins formed on incubation of chicken erythrocyte preparations with glycine, aminolævulic acid or porphobilinogen. Biochem. J. *63,* 87, 1956.

Lemberg, R. & Legge, J. W.: Hematin Compounds and bile pigments. Interscience Publishers, Inc. New York.

Nicholas, R. E. H. & Rimington, C.: Qualitative analysis of the porphyrins by partition chromatography. Scandinav. J. Clin. & Lab. Investigation *1,* 12, 1949.

Rappoport, D. A., Calvert, C. R., Loeffler, R. K. & Gast, J. H.: Chromatographic separation and determination of porphyrin methyl esters. Anal. Chem. *27,* 820, 1955.

Rimington, C.: Haem pigments and porphyrins. Ann. Rev. Biochem. *26,* 561, 1957.

dinav. J. Clin. & Lab. Investigation 323—332, 10, 1958.

STUDIES IN THE RESPONSE OF FREE AND CONJUGATED PLASMA 17-HYDROXYCORTICOSTEROIDS TO CORTICOTROPHIN IN MISCELLANEOUS CLINICAL DISORDERS [1,2]

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The determination of free plasma 17,21-ydroxy-20-ketosteroids (17-OHCS) after avenous ACTH stimulation is generally epted as a reliable test of adrenocortical acity (Eik-Nes, Sandberg, Nelson, Tyler Samuels, 1954; Bayliss & Steinbeck, 4; Christy, Wallace & Jailer, 1955). Abmalities in the response of plasma 17-ICS to intravenous ACTH have been erved in disorders of the adrenal cortex l pituitary, and in other clinical entities ik-Nes, Sandberg, Migeon, Tyler & Sa-els, 1955; Christy *et al.*, 1955, Sandberg, -Nes, Migeon & Samuels, 1956, Sand-g, Eik-Nes, Migeon & Koepf, 1957).

The object of the present investigation was study the applicability of the above-men-ned adrenocortical capacity test for clinical tine by performing the test on a group patients suffering from a variety of di-ses, mostly of non-endocrinologic origin. The determinations were extended to in-de also the conjugated plasma 17-OHCS, ich form a considerable portion of the l plasma 17-OHCS and reflect the meta-ism of the biologically active free steroids.

It has been reported (Gemzell & Franksson, 1953; Geller, Gabrilove & Soffer, 1957) that the achievement of plasma 17-OHCS levels at least equal in duration and degree to those obtained following the intravenous administration of ACTH is possible with a single intramuscular injection of a long-acting ACTH preparation. Therefore an attempt was made in a number of cases to use intramuscular ACTH administration instead of intravenous infusion.

MATERIAL AND METHODS

The series consisted of 61 hospital cases, in which the adrenocortical response to intravenous or intramuscular ACTH, or both, were studied. As there is a significant decrease in the rate of metabolic removal of plasma corticosteroids beyond 65 years of age (Samuels, Brown, Eik-Nes, Tyler & Dominguez, 1957), patients older than 65 years were excluded from this study. There was, however, one exception.

The intravenous ACTH infusion was started at 7—8 a.m. on fasting subjects. The blood samples were taken from the cubital vein immediately before the intravenous ACTH administration and 2 and 6 hours after the beginning of the test. The ACTH (ACTHAR, Armour Lab. or Cortrophine, Organon)[1] was given by continuous drip infusion in

Preliminary report presented at the Internaal Congress on Clinical Chemistry in Stockholm, . 1957.

Supported by a grant from the S:grid Jusélius telse.

[1] The ACTH preparations used in this investigation were generously supplied by the "Armour Lab." and "Organon".

normal saline or 5 per cent glucose solution. The ACTH dose used was 12—20 I. U. in 600—1000 ml of solution over a period of 6 hours. According to earlier investigations this is certainly sufficient to produce a maximal adrenocortical response (Bayliss & Steinbeck, 1954, Eik-Nes *et al.*, 1954).

A repository ACTH preparation (Cortrophine-Z, Organon) was used for intramuscular administration. A dose of 20 I. U. was given 3 or 4 times at intervals of 6 hours. In one case a single injection of 40 I. U. Cortrophine-Z was tried. The blood specimens were taken before the injections at 8 a. m. and 2—6 hours after the last injection.

The free plasma 17-OHCS were determined by Silber & Porter's method (1954) as modified by Peterson, Wyngaarden, Guerra, Brodie & Bunim (1955), and Peterson, Karrer & Guerra (1957). The free plasma 17-OHCS level at 8 a. m. has been averaged 12.7 ± 0.43 μg/100 ml with a range of 7—21 μg/100 ml for normal adults in this laboratory (Oka 1956).

To estimate the level of conjugated 17-OHCS the following procedure was used: 400 units of bacterial beta-glucuronidase (Sigma) in 1 ml of redistilled water, 0.5 ml of 1 M K_2HPO_4 and 0.3 ml of $NaHSO_3$, 5 per cent solution, were added to 5 ml of heparinized plasma and incubated at +37° C for 48 hours. The pH of the final mixture was 6.0—6.2. After the incubation the method was the same as for free plasma 17-OHCS. In this way the level of total plasma 17-OHCS was measured. To obtain the figure of conjugated plasma 17-OHCS the value of free 17-OHCS was subtracted from the value of total plasma 17-OHCS. Detailed data of the hydrolysis procedure for conjugated plasma 17-OHCS will be presented elsewhere (Krusius & Oka, 1958 a).

RESULTS

The response of plasma 17-OHCS to *intravenous* ACTH was determined in 50 cases. The adrenocortical capacity was considered as normal if the level of free plasma 17-OHCS after 6 hours ACTH infusion ranged from 30—48 μg/100 ml.

These normally reacting cases were divided into three groups according to the response of conjugated plasma 17-OHCS to ACTH. The conjugation and excretion mechanism of plasma 17-OHCS was regarded as normal if the levels of conjugated plasma 17-OHCS after 6 hours' ACTH stimulation ranged from 15—31 μg/100 ml, with a mean of 23 μg/100 ml (Table I). This was the case in 24 instances.

An increased response of conjugated plasma 17-OHCS to ACTH was observed in nine cases, in which the range of conjugated steroids at 6 hours was 37—79 μg/100 ml (Table II).

A decreased response of conjugated plasma 17-OHCS was seen in four cases in which the plasma levels of conjugated corticosteroids after 6 hours of ACTH infusion were 3, 5 and 8 μg/100 ml (Table III).

A hyperreactive adrenal cortex was found in ten cases. The levels of free plasma 17-OHCS after 6 hours of intravenous ACTH infusion ranged from 55—72 μg/100 ml in these patients (Table IV).

A hyporeactive adrenal cortex was observed only in two instances (Table V), one of these showing no reaction at all.

The ratio free/total plasma 17-OHCS was calculated. This ratio had a tendency to rise after 2 hours of ACTH infusion and to return to the initial value after 6 hours. Impairment of the conjugation or excretion of plasma 17-OHCS affected the ratio very clearly. It decreased with retarded renal clearance of the conjugated steroids and increased with defective conjugation.

The effect of *intramuscular* injections of Cortrophine-Z on plasma 17-OHCS levels is presented in Table VI. In the first case (M. By.) a single intramuscular dose of

Table I. *Normal adrenocortical capacity.*

	Age & sex	Diagnosis	Plasma 17 — OH — CS μg/100 ml								
			Before ACTH administration			After the onset of ACTH administration					
						2 hours			6 hours		
			Free	Conj.	Ratio free/ total	Free	Conj.	Ratio free/ total	Free	Conj.	Ratio free/ total
u.	51 F	Bronchial asthma	12	-	-	32	-	-	40	-	-
ä.	61 M	Hypertension, emphysema	14	-	-	29	-	-	38	-	-
e.	31 F	Hypertension[1]	15	14	0.52	28	15	0.65	37	24	0.61
o.	52 F	Congestive heart failure, Valvular disease	12	7	0.59	24	12	0.67	35	22	0.61
a.	65 F	Congestive heart failure, diabetes mellitus, chronic pyelocystitis	12	-	-	28	-	-	42	-	-
j.	60 M	Aortic aneurysm, chronic pulmonary tuberculosis	15	18	0.46	23	17	0.58	40	26	0.61
l.	50 M	Pulmonary tuberculosis addisonism	2	13	0.93	14	2	0.88	30	28	0.52
a.	33 F	Pulmonary tuberculosis, microcytic anemia	8	4	0.67	27	12	0.69	39	25	0.61
ä.	56 M	Acute nephrose-nephritis	6	10	0.38	29	8	0.77	48	22	0.69
e.	20 F	Bilateral hydronephrosis	19	-	-	31	-	-	37	-	-
o.	61 F	Biliary hepatic cirrhosis	14	10	0.59	28	9	0.76	41	15	0.73
i.	46 F	Obesity	12	8	0.60	28	12	0.70	33	25	0.57
s.	28 M	Diabetes mellitus	18	2	0.90	34	9	0.79	45	24	0.65
i.	59 F	Diabetes mellitus, polyneuritis, orthostatic hypotonia	12	8	0.60	31	10	0.76	42	18	0.70
a.	44 F	Hypothyroidism, duodenal ulcer	8	8	0.50	32	7	0.82	42	20	0.68
i.	39 F	Hypothyroidism, congestive heart failure, microcytic anemia	14	4	0.78	30	5	0.86	41	24	0.63
a.	57 F	Hyperthyroidism	22	11	0.67	33	18	0.65	48	25	0.66
i.	23 F	Hyperthyroidism	4	14	0.22	17	16	0.52	31	20	0.61
i.	39 F	Stein-Leventhal syndrome, choledocholithiasis	11	2	0.85	32	10	0.77	41	20	0.67
o.	41 M	Rheumatic fever	13	4	0.77	34	17	0.67	38	27	0.59
o.	34 M	Rheumatic fever	11	6	0.65	28	21	0.57	42	31	0.58
a.	45 M	Rheumatoid arthritis	15	3	0.83	33	13	0.72	42	23	0.65
a.	52 F	Rheumatoid arthritis	19	5	0.79	31	-	-	37	22	0.61
a.	49 F	Rheumatoid arthritis, hypertension, obesity	14	12	0.54	38	15	0.72	46	26	0.64
		Mean	12	8	0.64	29	10	0.71	40	23	0.63

[1] Creatinine clearance 84 ml/min/1.73 sq. m, phenolsulphonphtalein test 51.5 %.

Table II. *Normal adrenocortical capacity, but decreased renal clearance of conjugated 17,21-dihydro-20-ketosteroids.*

Case	Age & sex	Diagnosis	Plasma 17 — OH — CS μg/100								
			Before ACTH administration			After the onset of ACTH administration					
						2 hours			6 hours		
			Free	Conj.	Ratio free/ total	Free	Conj.	Ratio free/ total	Free	Conj.	Ratio free/ total
I. Uu.	52 F	Hypertension	19	14	0.58	41	13	0.71	43	44	0.5
H. Ha.	58 F	Hypertension,[1] benign nephrosclerosis	14	4	0.78	37	6	0.86	44	39	0.5
V. Hä.	26 M	Chronic nephritis	15	17	0.47	25	30	0.46	37	41	0.4
E. Sv.	53 F	Renal amyloidosis, rheumatoid arthritis[2]	22	30	0.43	31	42	0.42	37	59	0.3
P. Le.	69 M	Renal amyloidosis,[2] bronchiectasiae	16	42	0.28	25	48	0.34	33	76	0.3
M. Sv.	62 F	General amyloidosis, rheumatoid arthritis[3]	11	25	0.31	31	24	0.56	42	37	0.5
R. Le.	39 F	Chronic nephritis[2]	18	11	0.64	39	12	0.78	48	38	0.5
E. Lu.	50 M	Congestive heart failure, obesity, diabetes mellitus, bronchial asthma	8	20	0.29	31	43	0.42	43	79	0.3
S. Vi.	55 F	Obesity	17	16	0.52	34	31	0.52	45	44	0.5
		Mean	16	20	0.48	33	28	0.56	41	51	0.4

[1] Creatinine clearance 55—56 ml/min/1.73 sq. m. Phenolsulphonphtalein test 37.5—50.5 %.
[2] NPN increased, uremia. Creatinine clearance in case R. Le. 60 ml/min/1.73 sq. m and phenolsulphonphtalein test 46.5 %.
[3] Diagnosis confirmed at autopsy.

Table III. *Normal adrenocortical capacity, but decreased formation of conjugated 17,21-dihydro-20-ketosteroids.*

Case	Age & sex	Diagnosis	Plasma 17 — OH — CS μg/100 ml								
			Before ACTH administration			After the onset of ACTH administration					
						2 hours			6 hours		
			Free	Conj.	Ratio free/ total	Free	Conj.	Ratio free/ total	Free	Conj.	Ratio free/ total
K. Yl.	49 M	Toxoplasmosis	13	4	0.72	30	1	0.97	46	3	0.9
E. As.	42 F	Bronchitis, hypotension, general asthenia	17	0	1.00	38	0	1.00	40	5	0.8
U. Hi.	54 M	Congestive heart failure, emphysema	20	0	1.00	34	1	0.97	46	8	0.8
A. Ar.	30 F	Rheumatoid arthritis, chronic tonsillitis	9	0	1.00	23	5	0.83	35	8	0.8
		Mean	15	1	0.93	31	2	0.94	42	6	0.8

Table IV. *Hyperreactive adrenal cortex.*

	Age & sex	Diagnosis	Plasma 17 — OH — CS μg/100 ml								
			Before ACTH administration			After the onset of ACTH administration					
						2 hours			6 hours		
			Free	Conj.	Ratio free/ total	Free	Conj.	Ratio free/ total	Free	Conj.	Ratio free/ total
Kä.	46 F	Cushing's syndrome, pituitary adenoma	26	-	-	48	-	-	63	-	-
Dl.	57 F	Cushing's syndrome	25	15	0.63	56	25	0.70	72	59	0.55
Fu.	64 F	Hypertension, congestive heart failure	9	30	0.23	42	34	0.55	59	64	0.48
Si.	65 F	Hypertension, benign nephrosclerosis[1]	23	26	0.47	50	38	0.57	70	60	0.59
Si.	65 F	Renewed test 4 months later	22	17	0.57	41	14	0.75	57	32	0.64
Ra.	47 F	Hypertension, malignant nephrosclerosis[1]	18	22	0.45	44	18	0.71	68	40	0.63
'e.	62 F	Chronic nephritis, hypothyroidism[2]	29	8	0.79	46	9	0.84	67	27	0.71
My.	51 M	Congestive heart failure, hypertension, spondylarthritis anchylopoetica	20	16	0.56	43	16	0.73	57	25	0.70
Bä.	60 F	Congestive heart failure, hypertension, diabetes mellitus	12	11	0.52	43	17	0.72	60	27	0.69
Vu.	32 F	Diabetes insipidus[2]	16	4	0.80	44	12	0.79	55	26	0.68
My.	45 F	Rheumatic fever, erythema nodosum	20	13	0.61	42	22	0.65	55	25	0.69
		Mean	20	16	0.56	45	21	0.70	62	39	0.64

[1] Confirmed at autopsy.
[2] Creatinine clearance and phenolsulphonphtale'n test within normal ranges.

Table V. *Hyporeactive adrenal cortex.*

se	Age & sex	Diagnosis	Plasma 17 — OH — CS μg/100 ml								
			Before ACTH administration			After the onset of ACTH administration					
						2 hours			6 hours		
			Free	Conj.	Ratio free/ total	Free	Conj.	Ratio free/ total	Free	Conj.	Ratio free/ total
Vi.	44 M	Hyperthyroidism	13	10	0.57	22	12	0.65	26	22	0.54
He.	31 F	Addison's disease	0	0	-	0	0	-	0	0	-

Table VI. *Adrenocortical capacity test, performed by intramuscular injections of repository ACTH.*

Case	Age & sex	Diagnosis	ACTH dose I.U.	Number of injections	Interval of injections in hours	Interval after last injection in hours	Plasma 17 — OH — CS μg/100 ml					
							Before ACTH			After ACTH		
							Free	Conj.	Ratio free/ total	Free	Conj.	Ratio free/ total
M. By.	45 M	Healthy control	40	1	-	2	8	7	0.53	20	18	0.53
M. By.	45 M	Healthy control	40	1	-	4	8	7	0.53	25	40	0.39
M. By.	45 M	Healthy control	40	1	-	6	8	7	0.53	25	31	0.45
M. By.	45 M	Healthy control	40	1	-	8	8	7	0.53	18	59	0.23
M. By.	45 M	Healthy control	20	4	6	2	8	7	0.53	41	-	-
L. Pa.	45 F	Healthy control	20	3	6	6	12	10	0.55	32	21	0.60
A. Nu.	57 F	Convalescent (bone fracture)	20	3	6	4	12	17	0.41	45	33	0.58
A. Ah.	57 F	Convalescent (apoplectic stroke)	20	3	6	4	6	18	0.25	31	34	0.48
M. Ko.	48 F	Bronchial asthma	20	3	6	6	7	19	0.27	19	59	0.24
P. Ka.	34 F	Bronchial asthma	20	3	6	4	4	10	0.28	24	25	0.49
O. Ta.	55 M	Tuberculous spondylitis	20	3	6	6	14	11	0.56	52	42	0.55
V. Sa.	52 F	Herpes zoster	20	3	6	6	24	8	0.75	33	29	0.53
S. La.	60 F	Thrombophlebitis	20	3	6	6	13	8	0.62	39	27	0.59
V. Kä.[1]	46 F	Cushing's syndrome	20	3	6	4	20	9	0.69	45	-	-
K. Fo.	61 M	Pituitary adenoma	20	3	6	4	9	15	0.38	32	43	0.40
P. He.	52 M	Scleroderma, hypertension	20	3	6	4	10	7	0.59	30	22	0.58
M. La.[2]	52 F	Rheumatoid arthritis	20	3	6	6	11	9	0.55	11	15	0.42
		Mean					11	10	0.50	31	29	0.42

[1] Intravenous capacity test performed 3 months earlier (Table IV).
[2] Intravenous capacity test performed 10 days later (Table I).

40 I. U. did not effect the maximal adrenocortical response. The dosage was changed to 20 I. U., and 4 repeated intramuscular Cortrophine-Z injections were given. A maximal response of plasma 17-OHCS was now obtained. In twelve further cases 3 intramuscular injections of 20 I.U. Cortrophine-Z were given every six hours, and the blood samples were taken 4 or 6 hours after the last injection. The mean free plasma 17-OHCS level after intramuscular ACTH stimulation in this group was 31 μg/100 ml, which is 9 μg/100 ml less than in the group which received intravenous ACTH. One of the patients (M. La.) did not react at all to the intramuscular ACTH. The response of plasma 17-OHCS to intravenous ACTH was fully normal in this case.

DISCUSSION

The determination of the response of free plasma 17-OHCS to intravenous ACTH has proved to be a very sensitive test for adrenocortical capacity. This test is applicable to clinical routine and it has to a great extent replaced other adrenal function test in our hospital.

The maximal free plasma 17-OHCS levels re usually reached at the 6 hour after the set of the ACTH infusion, but the values four hours were in many instances not rkedly lower. A rise of the free plasma -OHCS to 30—48 μg/100 ml seemed to e good evidence of normal adrenocortical pacity.

In a large majority of the cases studied responsiveness of the adrenal cortex was rmal. Hyperreactive responses were found 3 cases suffering from diseases of adrenal hypophyseal origin, *i. e.* from Cushing's ndrome, pituitary adenoma with Cushing's ndrome and diabetes insipidus. One case th hyperreactive adrenal cortex had rheutic fever complicated by strong erythema dosum. All the remaining patients with perreactive adrenal cortex suffered from pertension (5), congestive heart failure) or renal failure (2), alone or combined. addition to this, increased adrenocortical pacity has previously been reported by one us in acute cases of tuberculous meningitis)ka, Österlund, Paatela & Wasz-Höckert, 57).

The mechanism causing increased activity the adrenal cortex in some cases of hypersion or congestive heart failure is not ite clear. In most of the cases the level of njugated plasma 17-OHCS was within rmal limits after 6 hours of intravenous CTH stimulation, thus showing no defect the conjugation or excretion of plasma ticosteroids. It seems reasonable to sugst that the adrenal cortex was really hyfunctioning in these cases. One of the ients (H. Ra.) with renal failure and reased response of free plasma 17-OHCS ACTH died and was autopsied. A distinct hypertrophy of the adrenal cortex could be demonstrated. In another case with hypertension and benign nephrosclerosis, however, autopsy did not reveal any changes in the size of the adrenal cortex (V. Si.).

A slightly hyporeactive cortex was found in a case of hyperthyroidism (Table V). This may have been due to an accelerated rate of steroid degradation and increased hydrocortisone requirement (Levin & Daughaday, 1955, Samuels *et al.*, 1957). However, the other thyrotoxic cases studied gave a normal response to intravenous ACTH. A patient with distinct clinical features of Addison's disease showed no response at all to ACTH. Other cases in which we observed a definitely hyporeactive adrenal cortex were those undergoing long-term corticosteroid treatment (Krusius & Oka, 1958 b).

The conjugated plasma 17-OHCS have not received much attention so far. In their normal series Brown, Englert, Wallach & Simons (1957) found a rise of conjugated plasma 17-OHCS from an average of 9 to 30 μg/100 ml after 6 hours of intravenous ACTH administration. In the study now reported the corresponding figures were somewhat lower, from 8 to 23 μg/100 ml. — A conjugation defect of plasma 17-OHCS has been reported in liver diseases (Bongiovanni, Eberlein, Grumbach, Van Wyck & Clayton, 1954; Klein, Papadatos, Fortunato, Byers & Puntereri, 1955; Englert, Brown, Wallach & Simons, 1957) and a retarded rate of clearance in renal failure (Klein, Papadatos, Fortunato & Byers, 1955; Samuels *et al.*, 1957). These findings were confirmed in this study. Six of the nine patients who showed normal adrenocortical capacity in the light of free plasma 17-OHCS, but

an increased response of conjugated steroids, were suffering from renal failure. The others were suffering from congestive heart failure, hypertension or obesity, but the possibility of impairment of renal function cannot be excluded because renal function tests were not performed. In four cases the conjugation of plasma 17-OHCS during ACTH infusion was delayed. One of these patients had toxoplasmosis affecting many organs, including the liver. In the remaining three cases no distinct signs of liver damage or dysfunction could be demonstrated.

In the ratio of free/total plasma 17-OHCS before and during intravenous ACTH stimulation slight increase occurred after two hours of ACTH administration, whereafter it returned to the initial level. This change indicates that the free plasma 17-OHCS level rises promptly in response to ACTH, but that the conjugation gets started somewhat later.

In the group of normal adrenocortical capacity and in the group of hyperreactive adrenal cortex the ratio free/total plasma 17-OHCS was about the same, thus showing that the conjugated plasma 17-OHCS generally reflect and follow changes in free plasma corticosteroids.

Low ratios free/total plasma 17-OHCS were found above all in renal failure. High ratios were observed in four cases, of which, however, only one showed distinct impairment of hepatic function.

An average of 63 per cent (range 52—70 per cent) of the plasma 17-OHCS appeared to be in free form after 6 hours of ACTH stimulation in cases with normal adrenocortical function.

We feel that the assay of conjugated or total plasma 17-OHCS in addition to plasma free 17-OHCS gives a more complete picture of the adrenocortical response to ACTH than the estimation of the free steroid fraction alone. The determination of conjugates seems to be of clinical value especially in cases of renal failure.

Our attempt to replace the intravenous method of ACTH administration by single or repeated intramuscular injections of repository ACTH preparations was not very successful. The response of plasma free 17-OHCS to intramuscular ACTH varied from case to case, and the levels reached were in many cases significantly lower than can be produced by intravenous ACTH stimulation of the adrenal cortex. The maximal effect of a single intramuscular Cortrophine-Z injection was measured after 4—6 hours. Thus the results do not corroborate the data presented in previous papers (Gemzell & Franksson, 1953; Geller *et al.*, 1957). The response of plasma conjugated 17-OHCS was, however, on the average stronger than in the intravenous capacity test and correspondingly the ratio free/total 17-OHCS was lower. This is evidently due to the longer period of adrenocortical stimulation in this series.

SUMMARY

Adrenocortical capacity tests by using 6 hours intravenous ACTH stimulation and measurement of plasma 17-OHCS levels were performed in 50 cases of miscellaneous medical disorders. The test proved to be a highly sensitive index of adrenocortical activity and to be applicable to clinical routine.

oth free and conjugated plasma 17-CS were estimated. Increased response he free plasma 17-OHCS to intravenous TH was recorded in 10 cases of Cush-'s syndrome, diabetes insipidus, rheumatic er, hypertension, congestive heart failure, l or renal failure. A slightly decreased ponse was measured in a case of hyper-roidism. One patient with Addison's dis-e showed no response.

An increased response of conjugated plas-17-OHCS to intravenous ACTH was n in 9 cases, 6 of which were those of al failure and the remaining cases those congestive heart failure, hypertension or sity. In 4 cases the response of conjugated sma 17-OHCS to ACTH was decreased. e of these patients suffered from toxo-smosis complicated by liver disease; the ers, however, showed no clinical evidence liver dysfunction.

After 6 hours of ACTH administration ratio free/total plasma 17-OHCS did not y noticeably from case to case and was out the same in the group with normal d increased adrenocortical capacity. A de-eased ratio was present in renal failure.

The adrenocortical capacity tests per-rmed by intramuscular application of re-sitory ACTH were not very successful. e response of plasma 17-OHCS to intra-uscular ACTH varied from case to case, d maximal levels were reached only in me of the cases.

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REFERENCES

Bayliss, R. I. S. & Steinbeck, A. W.: The adrenal response to corticotropin. Brit. Med. J. *1,* 486, 1954.

Bongiovanni, A. M., Eberlein, W. R., Grumbach, M. M., Van Wyck, J. J. & Clayton, G.: Conjugates of adrenal corticoids in human plasma. Proc. Soc. Exper. Biol. Med. *87,* 282, 1954.

Brown, H., Englert, E., Jr., Wallach, S. & Simons, E. L.: Metabolism of free and conjugated 17-hydroxycorticosteroids in normal subjects. J. Clin. Endocrinol. Metab. *17,* 1191, 1957.

Christy, N. P., Wallace, E. Z. & Jailer, J. W.: The effect of intravenously-administered ACTH on plasma 17,21-dihydroxy-20-ketosteroids in normal individuals and in patients with disorders of the adrenal cortex. J. Clin. Investigation *34,* 899, 1955.

Eik-Nes, K., Sandberg, A. A., Migeon, C. J., Tyler, F. H. & Samuels, L. T.: Changes in plasma levels of 17-hydroxycorticosteroids during the intravenous administration of ACTH. II. Response under various clinical conditions. J. Clin. Endocrinol. Metab. *15,* 13, 1955.

— — Nelson, D. H., Tyler, F. H. & Samuels, L. T.: Changes in plasma levels of 17-hydroxycorticosteroids during the intravenous administration of ACTH. I. A test of adrenocortical capacity in the human. J. Clin. Investigation *33,* 1502, 1954.

Englert, E. Jr., Brown, H., Wallach, S. & Simons, E. L.: Metabolism of free and conjugated 17-hydroxycorticosteroids in subjects with liver disease. J. Clin. Endocrinol. Metab. *17,* 1395, 1957.

Geller, J., Gabrilove, J. L. & Soffer, L. J.: Response of plasma 17,21-dihydroxy-20-ketosteroids to repository corticotropin. J. Clin. Endocrinol. Metab. *17,* 390, 1957.

Gemzell, C. A. & Franksson, C.: The effect of ACTH on the blood level of 17-hydroxycorticosteroids and the circulating eosinophils in man. Acta Endocrinol. *14,* 205, 1953.

Klein, R., Papadatos, C., Fortunato, J., Byers, C. & Puntereri, A.: Serum corticoids in liver disease. J. Clin. Endocrinol. Metab. *15,* 215, 1955.

— — — — Acid-hydrolyzable corticoids of serum. J. Clin. Edocrinol. Metab. *15,* 215, 1955.

Krusius, F.-E. & Oka, M.: Determination of conjugated 17-hydroxycorticosteroids in human peripheral blood. Scand. J. Clin. & Lab. Investigation *10,* 373, 1958 a.

— — Effect of long-term treatment with corticosteroids on adrenocortical function. Ann. Rheum. Dis. *17,* 184, 1958 b.

Levin, M. E. & Daughaday, W. H.: The influence of the thyroid on adrenocortical function. J. Clin. Endocrinol. Metab. *15,* 1499, 1955.

Oka, M.: Effect of cerebral vascular accident on the level of 17-hydroxycorticosteroids in plasma. Acta Med. Scandinav. *156,* 221, 1956.

— Österlund, K., Paatela, M. & Wasz-Höckert, O.: Adrenocortical capacity in the acute stage of tuberculous meningitis. Acta Endrocrinol. *24,* 402, 1957.

Peterson, R. E., Karrer, A. & Guerra, S. L.: Evaluation of Silber-Porter procedure for determination of plasma hydrocortisone. Anal. Chem. *29,* 144, 1957.

Peterson, R. E., Wyngaarden, J. B., Guerra, S. L., Brodie, B. B. & Bunim, J. J.: The physiological disposition and metabolic fate of hydrocortisone in man. J. Clin. Investigation *34,* 1779, 1955.

Samuels, L. T., Brown, H., Eik-Nes, K., Tyler, F. H. & Dominguez, O. V.: Extra-adrenal factors affecting the levels of 17-hydroxycorticosteroids in plasma. Ciba Foundation Colloquia on Endocrinology, Vol. 11. Hormones in Blood. J. & A. Churchill LTD 1957.

Sandberg, A. A., Eik-Nes, K., Migeon, C. J. & Koepf, G. F.: Plasma 17-hydroxycorticosteroids in hyperfunction, suppression and deficiency of adrenal cortical function. J. Lab. Clin. Med. *50,* 286, 1957.

— — — & Samuels, L. T.: Metabolism of adrenal steroids in dying patients. J. Clin. Endocrinol. Metab. *16,* 1001, 1956.

Silber, R. H. & Porter, C. C.: The determination of 17,21-hydroxy-20-ketosteroids in urine and plasma. J. Biol. Chem. *210,* 923, 1954.

ndinav. J. Clin. & Lab. Investigation 333—336, 10, 1958.

EFFECT OF CORTICOTROPHIN ON PLASMA CHOLINESTERASE ACTIVITY

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The activity of plasma cholinesterase hE) has been studied extensively in a riety of diseases. The enzyme is probably nthesized in the liver (Sawyer & Everett, 47), and a decrease in its plasma level urs especially in liver damage (Antopol, chman & Schifrin, 1938).

There are, however, conditions in which fall in plasma ChE activity cannot be atbuted to a defect in the synthesis of the zyme. Certain acute conditions, such as rns (Heim & Ruete, 1946; Schümmeler, 1946), traumatic shock (Frommel, alheimer, Herschberg & Piquet, 1943; on & Herschberg, 1947; Laborit & Moid, 1947), surgical operations (Snyder, yder & Bunch, 1951), and myocardial inction (Oka, 1954 a; Moore, Birchall, rack & Botson, 1957) are followed by a rked fall of plasma ChE activity. All these tes belong to the category of "stress", aracterized by an increased level of plasma rticosteroids (Sandberg, Eik-Nes, Samuels Tyler, 1954; Engel, Helmreich, Hertogh, bre, Muller & Mach, 1955; Oka, 1956 a,b). suggestion that the decrease of plasma E in the above-mentioned disorders is e to an increased adrenocortical function l the inhibitory effect of the elevated sma corticosteroids on the ChE activity is ipting.

This problem has been studied and discussed in some earlier papers, but the results are contradictory. Maier (1953) administered cortisone to 4 patients with various diseases and recorded a decrease in serum ChE activity. On the other hand, cortisone *in vitro* has no effect on serum ChE activity (Maier, 1953; Oka, 1954 b). Kaufman (1954) reports two cases in which serum ChE activity decreased after a single intramuscular injection of 25 mg of ACTH.

Clinical studies indicate that the concentration of plasma corticisteroids (17-OHCS) cannot affect plasma ChE (Oka, 1954 b). In rheumatic fever and in active lupus erythematosus, in which a marked decrease of plasma ChE activity occurs, an increase and normalization of the enzyme activity has been demonstrated during cortisone treatment simultaneously with clinical improvement. The changes in plasma ChE have been similar in cases treated with cortisone and salicylates and related to the changes in the clinical condition of the patient. In a few cases of rheumatoid arthritis treated with cortisone, plasma ChE activity did not change during the treatment.

The aim of the investigation here reported was to answer the question whether plasma ChE activity is directly affected by the plasma corticosteroid level or not. For this pur-

pose simultaneous determinations of plasma ChE activity and 17,21-dihydroxy-20-keto-steroids were performed before and during the course of a 6-hour intravenous ACTH drip infusion.

MATERIAL AND METHODS

The series consisted of 5 unselected cases in which a usual intravenous adrenocortical capacity test was carried out. The technique was identical with that used in our previous investigations (Krusius, Oka & Kärkela, 1958). Blood specimens were drawn before and 2, 4 and 6 hours after the beginning of ACTH administration and analyzed for plasma free and conjugated 17-OHCS and ChE activity. ChE activity was always determined immediately; the 17-OHCS assays were usually started on the same or following day.

Plasma ChE activity was determined by the method of Michel (1949). The method of Silber & Porter, modified by Peterson, Karrer & Guerra, 1957), was used for the assay of plasma free and conjugated 17-OHCS. The hydrolysis of 17-OHCS conjugates was done using a procedure of our own (Krusius & Oka, 1958). To estimate the normal liver function, routine liver function tests including the determination of serum glutamic-oxaloacetic transaminase activity were carried out. The results were within normal ranges in all instances, thus revealing no signs of liver damage.

RESULTS AND DISCUSSION

The results are briefly summarized in the Table. Attention was paid to the plasma levels of free and conjugated 17-OHCS and of ChE activity only. Three of the cases showed a normal adrenocortical response to ACTH stimulation, whereas there was a hyperreactive response in the two remaining cases. The plasma ChE activity was within normal ranges in all instances before the administration of ACTH; only in one case was the activity slightly augmented, but still within normal limits. This patient (E. Sv.) suffered from renal amyloidosis and hypertension complicated by uremia.

Independently of the adrenocortical response in the individual cases, the plasma ChE activity was not significantly altered by the stimulation of the adrenal cortex with ACTH, nor did the values after 2, 4 and 6 hours of ACTH administration differ from each other more than can be explained by diurnal or methodical variations.

Our results demonstrate clearly that stress as such — indicated by a marked elevation of plasma 17-OHCS values — has no direct effect on plasma ChE activity. In burns, traumatic shock, surgical treatment, myocardial infarction and other primary tissue lesions in which the plasma ChE activity is reported to decrease, this decrease is apparently not caused by a hyperfunction of the adrenal cortex. There must be some other explanation. Theoretically stress could produce damage of the liver and consequently a decrease of plasma ChE activity. However, the change of the ChE activity in the above-mentioned injuries is so rapid that a diminished enzyme formation in the liver cannot be responsible for it, because the biological half-life of pseudocholinesterase is supposed to be about 28 days (Sawyer & Everett, 1947). At present the most plausible explanation of this phenomenon seems to be that in tissue injuries, substances which have an inhibitory effect on the plasma ChE activity are released into circulation. Studies on the occurrence of inhibitors of cholinesterase activity in acute clinical conditions with tissue necrosis are in progress.

Table I. *Plasma free and conjugated 17-OHCS and cholin esterase activity during adrenocortical capacity test.*

ase, age sex	Diagnosis	Before ACTH administration		After ACTH administration					
				2 hrs.		4 hrs.		6 hrs.	
		Free/Conj. 17-OHCS µg/100 ml	ChE Δ pH/hr.	Free/Conj. 17-OHCS µg/100 ml	ChE Δ pH/hr.	Free/Conj. 17-OHCS µg/100 ml	ChE Δ pH/hr.	Free/Conj. 17-OHCS µg/100 ml	ChE Δ pH/hr.
My. F	Rheumatic fever, erythema nodosum	20/13	0.60	42/22	0.62	50/28	0.54	55/25	0.55
Ar. F	Rheumatoid arthritis	9/ 0	0.66	23/ 5	0.62	30/12	0.62	35/ 8	0.70
Sv. F	Rheumatoid arthritis, renal amyloidosis [1]	22/30	0.85	31/42	0.85	31/46	0.84	37/59	0.90
Si. F	Hypertension, nephrosclerosis [2]	22/17	0.68	41/14	0.59	49/24	0.66	57/32	0.69
Le. F	Chronic nephritis [3]	18/11	0.64	39/12	0.72	51/20	0.69	49/38	0.58
Mean		18/14	0.69	37/19	0.68	42/26	0.67	47/32	0.68

[1] NPN 107 mg per 100 ml.
[2] Diagnosis confirmed by autopsy.
[3] Creatinine clearance 60 ml/min/1.73 sq.m, PSP-excretion 46.5 %/2 hrs. NPN mg per cent.

SUMMARY

Stimulation of the adrenal cortex by means intravenous corticotrophin infusions, which sulted in elevated plasma free and con-gated 17,21-dihydroxy-20-ketosteroid le-ls, is shown to have no effect on plasma olinesterase activity.

REFERENCES

topol, W., Tuchman, L. & Schifrin, A.: Decreased cholinesterase activity of serum in jaundice and in biliary disease. Proc. Soc. Exper. Biol. Med. *38,* 363, 1938.

on, E. & Herschberg, A. D.: Choc anaphylactique et cholinestérase sérique. Presse Med. *55,* 314, 1947.

gel, E., Helmreich, M. L., Hertogh, J., Fabre, J., Muller, A. E. & Mach, R. S.: Variations du taux plasmatique des 17-hydroxycorticostéroides dans l'agression aiguë en clinique. Sem. hôp. Paris *31,* 78, 1955.

Frommel, E., Thalheimer, M., Herschberg, A. D. & Piquet, J.: Choc traumatique et cholinestérase sérique. Helv. physiol. pharmacol. Acta *1,* 451, 1943.

Heim, F. & Ruete, A.: Serumcholinesterasewerte und Blutzuckerwerte bei einigen, insbesondere allergiebedingten Hauterkrankungen. Klin. Wchnschr. *24—25,* 86, 1946.

Kaufman, K.: Serum cholinesterase activity in the normal individual and in people with liver disease. Ann. Int. Med. *41,* 533, 1954.

Krusius, F.-E. & Oka, M.: Determination of conjugated 17-hydroxycorticosteroids in human peripheral blood. Scandinav. J. Clin. & Lab. Investigation *10,* 373, 1958.

— — & Kärkelä, A.: Studies in the response of free and conjugated plasma 17-hydroxycorticosteroids to corticotrophin in miscellaneous clinical disorders. Scandinav. J. Clin. & Lab. Investigation *10,* 323, 1958.

Laborit, H. & Morand, P.: Choc traumatique et cholinestérase. Mém. Acad. Chir. (Paris) *73,* 326, 1947.

Maier, E. H.: Über den aktivitätsmindernden Einfluss des Cortisons auf die menschliche Serumcholinesterase in vivo. Klin. Wchnschr. *31,* 1008, 1953.

Michel, H. O.: An electrometric method for the determination of red cell and plasma cholinesterase activity. J. Lab. Clin. Med. *34,* 1564, 1949.

Moore, C. B., Birchall, R., Horack, H. M. & Batson, H. M.: Changes in serum pseudocholinesterase levels in patients with disease of the heart, liver or musculoskeletal systems. Amer. J. Sci. *234,* 538, 1957.

Oka, M.: Some observations on the cholinesterase activity of plasma in myocardial infarction. Acta Med. Scandinav. *150,* 313, 1954 a.

— Studies on the cholinesterase activity of red cell, plasma and synovial fluid with special reference to rheumatic diseases. Acta Med. Scandinav. Suppl. *293,* 1954 b.

— Effect of cerebral vascular accident on the level of 17-hydroxycorticosteroids in plasma. Acta Med. Scandinav. *156,* 221, 1956 a.

— Effect of myocardial infarction and stenocardia on the level of 17-hydroxycorticosteroids in plasma. Acta Med. Scandinav. 156, *227,* 1956 b.

Peterson, R. E., Karrer, A. & Guerra, S. L.: Evaluation of Silber-Porter procedure for determination of plasma hydrocortisone. Anal. Chem. *29,* 144, 1957.

Sandberg, A. A., Eik-Nes, K., Samuels, L. T. & Tyler, F. H.: The effects of surgery on the blood levels and metabolism of 17-hydroxycorticosteroids in man. J. Clin. Investigation *33,* 1509, 1954.

Sawyer, C. H. & Everett, J. W.: Cholinesterases in rat tissues and the site of serum non-specific cholinesterase production. Amer. J. Physiol. *148,* 675, 1947.

Schümmelfeder, N.: Der Einfluss örtlicher Durchblutungsstörungen auf die Cholinesterase des Blutes. Klin. Wchnschr. *24—25,* 113, 1946.

Silber, R. H. & Porter, C. C.: The determination of 17,21-dihydroxy-20-ketosteroids in urine and plasma. J. Biol. Chem. *210,* 923, 1954.

Snyder, H. E., Snyder, C. D. & Bunch, L. D.: Serum cholinesterase levels in surgical patients; a preliminary report. Amer. Surgeon *17,* 959, 1951.

dinav. J. Clin. & Lab. Investigation 337—343, 10, 1958.

OLYSACCHARIDE CONTENT OF SERUM IN PATIENTS WITH HYPERGAMMAGLOBULINEMIA

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n an earlier investigation (Laurell, Lau- & Waldenström, 1957) the polysaccha- content of serum and of its electrophor- fractions was found to differ widely in eloma and in macroglobulinemia. For parison in the present paper the corre- nding values are given for sera from ents with another type of hypergamma- oulinemia. This type is readily distin- shed electrophoretically from myeloma macroglobulinemia by the diffuseness of γ-globulin band and the more or less ked increase of the major components he γ-region. In unselected clinical material majority of all cases of hypergamma- oulinemia belong to this group. This type been observed in sera from patients with acute and chronic infections, collagenosis, umatoid arthritis, hepatic cirrhosis, tu- rs, Hodgkin's disease, sarcoidosis, leuke- s, Hashimoto's disease, Kimmelstiel- lson's syndrome and other diseases.

Seibert, Pfaff and Seibert (1948) exa- ed the hexose content of γ-globulins iso- d from sera from healthy subjects and nd it to be 1.6 per cent. Later investiga- (cf. Goa, 1955; Müller-Eberhard & nkel, 1956) have reported values ranging ween 1.2 and 3.2 per cent. This wide ge may possibly be ascribed to methodo- logical differences. These authors found either relatively high or relatively low values throughout for normal sera as well as for a few sera examined from patients with hypergammaglobulinemia of the types seen for example, in liver cirrhosis, systemic lupus and rheumatoid arthritis. The γ-globulins have usually been obtained by preparative electrophoretic separation. Müller-Eberhard & Kunkel (1956) have shown that part of the fast γ-globulins consists of a component with a larger hexose content (about 6 per cent) and a higher sedimentation constant (S19) than the bulk of the γ-globulins (S7), which usually contain about 1.2 per cent hexose. This implies that the hexose content of the total γ-globulin fraction will vary with the ratio between these two main components. The amounts of hexose, hexosamine and sialic acid in the γ-globulins have been determined by Schultze (1955), who found 1.3 per cent, 0.5 per cent and 0.3 per cent, respectively. He found a lower hexosamine content and a higher sialic acid content than reported by Müller-Eberhard & Kunkel. The hexosamine content of γ-globulins isolated from sera from healthy subjects and from a few patients with hypergammaglobulinemia has been reported by Boas & Reiner (1951), Goa (1955), Bollet (1957),

Sonnet (1956) and Südhof (1957). No significant difference between the hexosamine content of the γ-globulins of the controls and the patients were reported in these small series. Schaffner, Scherbel & Sytte (1956), Stidworthy, Payne, Shetlar & Shetlar (1957) and Stary (1957) found the same relative polysaccharide content of the γ-globulins in normal and hypergammaglobulinemia sera, while Puls & Fiedler (1956), who followed the relative polysaccharide content of the γ-fraction during acute exacerbations of hepatic cirrhosis, found transient increases. H. Laurell (1956) has found the hexose : protein ratio in the γ-globulins to be increased in patients with rheumatoid arthritis. Weimer, Redlich-Moshin & Nelson (1955) found immunisation to increase the hexose : protein ratio in γ-globulins in rabbits.

The results reported concerning the polysaccharide content of the γ-globulins from patients with hyper-γ-globulinemia are thus partly contradictory.

MATERIAL

The material was divided into four main groups, hepatic cirrhosis, systemic lupus, kala-azar and miscellaneous :

Hepatic cirrhosis (22 cases). — The diagnosis was regularly made on the basis of the patient's history, clinical examination and laboratory studies. Five patients had a history of acute or subacute hepatitis; six were heavy drinkers and one had had transfusion hepatitis. In most cases the diagnosis was verified by histologic examination of biopsy specimens or post mortem.

Systemic lupus (14 cases). — The diagnosis was made mainly on the basis of the patient's history and clinical findings. Positive L. E. cells were found in seven of the cases. In one of the other cases the diagnosis was confirmed at post mortem examination. The earlier patients were not regularly examined for L. E. cells, nor were they all examined during an exacerbation.

Kala-azar. — Sera from six typical patients were kindly supplied by Dr. Das Gupta, Bombay.

Miscellaneous (20 cases). — This was a fairly mixed group and included cases of cancer, lymphosarcoma, leukemia, "essential" hypergammaglobulinemia, chronic infections, diabetes *etc.*

METHODS

The analytical methods and the basis for the computations have been published earlier (Laurell Laurell & Waldenström, 1957, Laurell, 1958).

RESULTS AND DISCUSSION

Electrophoretic protein values vary with the technique employed. Therefore we have compared the values obtained from the patients with those from healthy blood donors using the same technique. All of the 62 sera studied contained more than 1.8 g γ-globulin per 100 ml, which was more than twice the value found for the controls (M = 0.87).

The electrophoretic distribution of the proteins in sera from patients with hepatic cirrhosis and with systemic lupus is given in Table I together with the normal values.

In the cirrhosis group he disease was in general well advanced. The means found for albumin and α_2-globulin were accordingly well below normal.

In the patients with systemic lupus the albumin levels were also depressed, but not markedly so. The α-values showed a wide range with levels well above and well below the normal limits. This scatter is probably due to differences in the phase of the disease at the time the specimens were collected. During an acute attack the albumin value fall, usually with an accompanying increase in the α-globulins above normal, unless the disease process includes a hemolytic component.

Table I. *The electrophoretic protein distribution in 50 normal persons (N), 22 cases with cirrhosis of the liver (C) and 14 cases with systemic lupus (L.e.d.). All values are given as g per 100 ml serum.*

		Total protein	Alb.	α_1	α_2	β	γ
Mean value	N	7.1	4.5	0.34	0.53	0.85	0.87
	C	7.3	2.7	0.32	0.42	0.94	2.98
	L.e.d.	8.7	3.5	0.39	0.64	0.83	3.25
S. D.	N	0.26	0.20	0.036	0.051	0.06	0.11
	C	0.99	0.70	0.062	0.098	0.19	0.74
	L.e.d.	1.05	0.74	0.10	0.20	0.20	1.16
Highest value	C	9.0	4.5	0.41	0.61	1.35	4.20
	L.e.d.	10.4	4.5	0.66	1.20	1.25	5.78
Lowest value	C	5.5	1.6	0.17	0.23	0.67	1.89
	L.e.d.	6.3	2.0	0.27	0.44	0.53	1.81

n the patients with cirrhosis the concention of hexose in α_1-globulin was on the rage 5.3 per cent and in systemic lupus per cent, the corresponding figures for being 5.8 and 7.3 per cent.

This suggests that the decrease in the α-ues in liver cirrhosis is due to a decrease the concentration of α-components richest polysaccharides. The reduction in the ount of hexose in the α_1-fraction can probly be attributed to a decrease in the acid glycoprotein and the decrease in the xose content of α_2 can be ascribed mainly a reduction in the haptoglobins which rich in polysaccharide. In liver cirrhosis "mucoproteins" (Winzler, 1955) are deased and in systemic lupus normal or inased. This fits in well with the α-changes erved after electrophoretic analysis with .S. staining[1] (Table II). In the individual es, however, the total serum polysacchari- (hexose + hexosamine + sialic acid) were only poorly correlated with the α-values, especially in cirrhosis. In these patients the polysaccharide content of the serum was found to be normal despite the low concentration of the α_1 and α_2-fractions normally richest in polysaccharide. This may tentatively be explained by the abnormally high concentration of polysaccharides in the γ-fraction. In serum the main carriers of hexose, hexosamine and sialic acid are the α_1- and α_2-globulins. In liver cirrhosis these fractions are decreased, but the serum levels of hexose, hexosamine and sialic acid levels are high. Therefore these three components must be present in increased absolute amount in the γ-fraction. The similarity in the proportion of hexose, hexosamine and sialic acid, despite differences in the α-values (Table III) in the three groups studied, suggests that the γ-globulin fractions in these pathological sera contain hexose, hexosamine and sialic acid in approximately the same proportion as the predominant glycoproteins.

[1] P.A.S. = periodic acid Schiff.

Table II. *The concentration of different polysaccharide components in serum from 34 normal (N), 22 cases with cirrhosis of the liver (C) and 14 cases of systemic lupus (L.e.d.). The electrophoretic hexose distribution has been calculated on the values for serum hexose and on values obtained after P. A. S.-staining of electrophoretic strips. All values are given as mg per 100 ml serum.*

		Protein-bound polysaccharide components			Calculated hexose content in the different electrophoretic fractions				
		Hexose	Hexos-amine	Sialic acid	Alb.	α_1	α_2	β	γ
Mean value	N	119	86	61	10	25	44	30	10
	C	144	117	62	9	18	27	38	52
	L.e.d.	180	139	75	8	34	50	36	52
S. D.	N	7	5	5	2.5	3.5	4.4	5.0	3.2
	C	21	14	9	3.6	5.4	6.6	9.6	11.4
	L.e.d.	21	14	15	6	11.5	15.5	9.7	15.7
Lowest value	C	109	91	45	2	8	17	19	37
	L.e.d.	147	115	53	2	20	33	21	25
Highest value	C	177	145	76	15	27	40	57	73
	L.e.d.	219	169	102	20	52	85	59	76

Table III. *Mean values for the relative proportions between hexose: hexosamine: sialic acid.*
The hexose values have been used as the basis for the calculation. The number of sera analysed are given in brackets.

	Hexose	Hexos-amine	Sialic acid
Liver cirrhosis	2 (22)	1.61 (19)	0.89 (17)
Systemic lupus	2 (13)	1.56 (13)	0.89 (10)
Miscellaneous	2 (15)	1.56 (15)	0.82 (15)

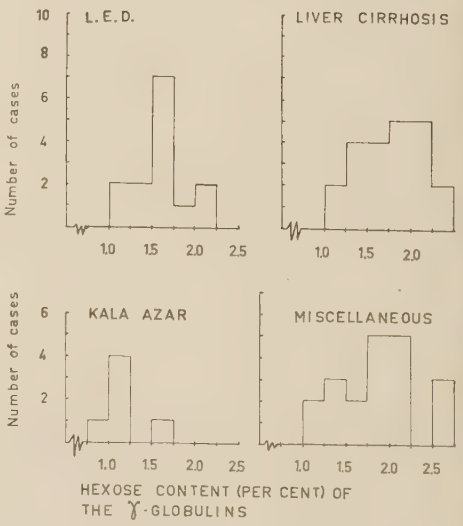
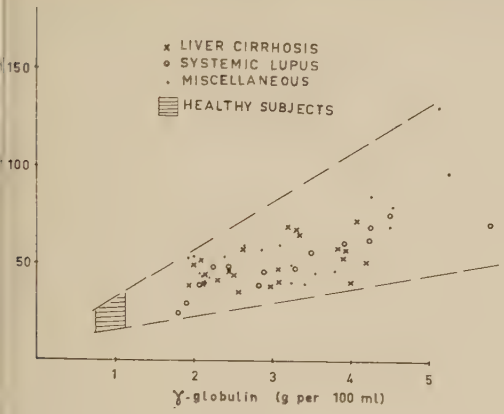


Fig. 1. Hexose content of γ-globulin in various diseases.

Fig 1 shows the approximate hexose content of the γ-fraction in patients belonging to the various groups. Some of the cases showed a hexose value of 1 to 1.5 per cent, *i. e.* a range similar to that of the bulk of the normal γ-globulins determined by this technique. The scatter in each group was considerable. Values above 2 per cent were noted in the three main groups. This cannot be ascribed entirely to the inaccuracy of



. 2. Correlation between hexose and protein in the γ-globulin fraction.

method. No close correlation was found ween the size of the γ-globulin fraction l its absolute content of hexose (Fig. 2) of polysaccharides. The range of variation the hexose content of the fraction was wide in mild as in severe hypergamma-bulinemia. This variation may be tentatively explained in different ways. The rmal electrophoretic γ-fraction contains relatively fast component with a sedimentation constant of 19 S. This component contains 6 per cent hexose (Müller-erhard & Kunkel, 1956). The assumption t variations in the amount of this component might be mainly responsible for the variation in the hexose content of the γ-fraction supported by the observation that a normal xose content (0.8—1.5 per cent) was found sera from the patients with kala-azar. rther their protein content was increased y within the field of the slow and medium globulins but not in the region of the fast globulins, where the 19 S components are be found. Ultracentrifugation of these sera vealed no demonstrable increase in the normal 19 S components, despite the fact that the Sia water dilution test was positive.

On electrophoresis of serum with subsequent P.A.S. staining, the polysaccharides sometimes stained more intensely than would be expected from the protein content in the zone of the fast γ-globulins, suggesting that the fast globulins contained relatively more polysaccharide than the slow γ-globulins. This relative increase in the intensity with which the polysaccharides stained was more common among the cases of alcoholic cirrhosis than among the cases of systemic lupus and posthepatitic cirrhosis. The results might be ascribed to a formation of 7 S γ-globulins with an abnormally high polysaccharide content or to an increase of the 19 S fraction. Those with the highest concentration of polysaccharide should theoretically be found among the fast globulins, since sialic acid increases the mobility of the component. This point will be the subject of a future paper.

CLINICAL DISCUSSION

Four short case histories are reported since the γ-fraction of these patients had the highest polysaccharide content of the whole material (Fig. 2, group miscellaneous). The history of the patient with the highest hexose content has already been published by one of the authors as case 3 in Waldenström (1955). He suffered from a very severe allergy and also showed signs of widespread vascular disease. The effect of cortisone treatment was dramatic. His body weight started to increase without accumulation of edema. His azotemia became normal. The serum albumin increased and the γ-globulin values decreased. This amelioration lasted about one year. He then became increasingly dyspnoic and died. The post-mortem showed signs of wide-spread polyangeitis nodosa and of encephalomalacia.

Another case was a man born in 1903. He had suffered from slight purpura and had an enormous

spleen, a very high sedimentation rate with a broad γ-globulin. His hematologic status showed slight leukopenia and some relative lymphocytosis. His chief complaint was persistent diarrhoea. The spleen was removed in 1956, when the patient had had a high sedimentation rate for at least 9 years. There were found numerous lymph glands retroperitoneally. One of these was extirpated with the spleen. The histological picture in both organs was interpreted as indicating lymphatic leukemia. After the operation the patient felt better for six months. Afterwards he had new periods of diarrhoea but less severe. His purpura has also improved. In 1958 the patient has had several purpuric periods.

Also another serum comes from a patient with lymphatic leukemia. This serum contained only traces of α-globulins. We have not had an opportunity to examine the patient but the clinical diagnosis was definitely lymphatic leukemia according to the doctor who has taken care of the patient.

The fourth patient suffered from what was clinically regarded as aplastic anemia for many years. Sternal puncture however showed a very cellular marrow with the possibility of myeloid leukemia. She was given very considerable quantities of blood (190 transfusions) and developed signs of secondary hemosiderosis. Her γ-globulin value was high. This was never really explained when the patient died. She was found to have wide-spread hemosiderosis in most organs with reactive portal cirrhosis. The post mortem confirmed the diagnosis myeloid leukemia. The protein disturbances may have been caused either by the hematologic process or by the liver cirrhosis.

This history has already been published as an instance of purpura hyperglobulinemica. The patient, who had had a high sedimentation rate for 15 years and had shown signs of inoperable gastric tumor, later died and was found to suffer from lymphosarcoma ventriculi. It seems probable that the tumor cells in the very massive gastric neoplasm had produced the pathological γ-globulins.

The variation observed in the polysaccharide content of the γ-fraction in the types of hyperglobulinemia studied does not appear to be bound to any particular disease and thus yields no information of differential diagnostic value. The variations may rather reflect different phases of the same disease or individual differences in the response of the γ-globulin producing cells (organic or genetic differences).

SUMMARY

The electrophoretic distribution of protein and protein-bound carbohydrates in sera from 62 patients with different types of hypergammaglobulinemia (hepatic cirrhosis, systemic lupus, kala-azar, and other diseases) are described. The relative polysaccharide content of the γ-fraction of these sera was found to vary between normal and twice normal value. The possible cause of this variation is discussed.

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REFERENCES

Boas, N. F. & Reiner, M.: Effect of ACTH and cortisone on serum hexosamine and gammaglobulin levels in acute disseminated lupus erythematosus. J. Clin. Endocrinol. *11,* 890, 1951.

Bollet, J. A., Rozhin, J. & Simpson, W.: The hexosamine content of the serum of globulins in normal and pathological sera. J. Clin. Investigation *36,* 51, 1957.

Goa, J.: On protein-bound carbohydrates in human serum. Scandinav. J. Clin. & Lab. Investigation *7,* suppl. 22, 1, 1955.

Laurell, C.-B., Laurell, H. & Waldenström, J.: Glycoproteins in serum from patients with myeloma, macroglobulinemia and related conditions. Am. J. Med. *22,* 24, 1957.

Laurell, H.: A paper electrophoretic study of the effect of ACTH and cortisone on the protein-bound serum polysaccharides in some patients with rheumatoid arthritis, ulcerative colitis etc. Ciba Foundation Symposium on Paper Electrophoresis 1956, p. 58.

rell, H.: Determination of hexosamine. p. 50 in Studies on preparative vertical zone electrophoresis. Almqvist & Wiksells Ltd. Uppsala 1958.

, W. & Fiedler, L.: Über die Polysaccharidgehalt der Serumeiweissfraktionen bei Gesunden und Kranken. Clin. Chim. Acta *1,* 348, 1956.

ler-Eberhard, H. J. & Kunkel, H.: The carbohydrate of γ-globulin and myeloma protein. J. Exper. Med. *104,* 253, 1956.

affner, F., Scherbel, A. L. & Lytle, R.: Electrophoretic serum glycoproteins in acute viral hepatitis. J. Lab. Clin. Med. *48,* 551, 1956.

ultze, H. E. & Biehl, H.: Vorschläge zur Methodik der präparativen Zonenelektrophorese auf Filterpapier, ihre Anwendungsmöglichkeiten und -grenzen bei der Trennung von Serumproteinen. Behringwerk-Mitteilungen *30, 18,* 1955.

ert, F. B., Pfaff, M. L. & Seibert, M. V.: Serum polysaccharide in tuberculosis. Arch. Biochem. *18,* 279, 1948.

net, J.: Les glycoprotéines sériques à l'état normal et pathologique. Editions Arscia. Bruxelles 1956.

Stary, Z.: Mucoproteins in clinical chemistry. Clin. Chem. *3,* 557, 1957.

Stidworthy, G., Payne, R. W., Shetlar, C. L. & Shetlar, M. R.: Objective evaluation of patients with rheumatic diseases. II. Paper electrophoretic studies of serum glycoprotein and protein from patients with rheumatoid arthritis. J. Clin. Investigation *36,* 309, 1957.

Südhof, H. & Kellner, H.: Physiologie und klinische Bedeutung kohlehydrathaltiger Körperstoffe. Bibl. paediat. No. 65, 1957.

Waldenström, J.: Some clinical aspects of the diagnosis and treatment of vascular allergy. Transactions of the Collegium Internationale Allergologicum, Second Symposium in Basel. July 1955.

Weimer, H., Redlich-Moshin, J. & Nelson, E.: Comparative effects of infection and immunization on serumglucoprotein concentrations. J. immunol. *74,* 243, 1955.

Winzler, R. J.: Determination of serum glycoproteins. Methods of biochemical analysis, *2,* 279, 1955.

Technique and Practical Problems

Scandinav. J. Clin. & Lab. Investigation 344, 10, 1958.

AN APPARATUS FOR AUTOMATIC COLORING AND DE-COLORING OF ELECTROPHORESIS PAPERS

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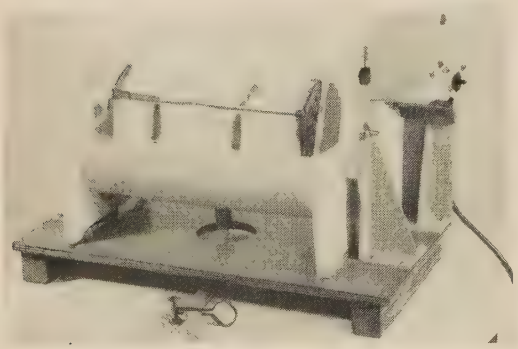


Fig. 1.

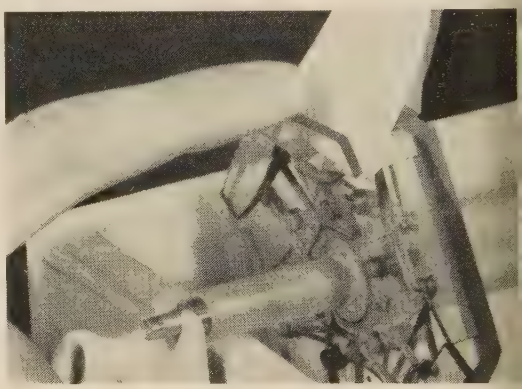


Fig. 2.

Coloring and de-coloring of electrophoresis papers are time and labor consuming. In order to simplify these procedures an apparatus has been constructed (Fig. 1). It consists of:

1. A framework made of plastic to which the paper strips can be fastened. The framework rests on support bearing and rotates freely. It consists of a central axis with two hexagonal plates and in the one end a large wheel. T-formed plastic pieces are fastened to the hexagonal plates by means of rubber bands. The T-formed pieces can be moved about one cm from the plates when fastening the end of the electrophoresis paper (Fig. 2).

2. A trough of ceramic in which the framework can rotate. The trough is furnished with two hollow taps, one on the end side and one in the bottom. These taps serve as support for rubber tubes used for filling and emptying the trough.

3. An electric motor built in a metal case. The motor either rests backwards in a "free position" or rests with its wheel on the large wheel. The surface of the motor-wheel is covered with rubber in order to establish sufficient friction between the two wheels. A convenient speed of rotation is 6 times per minute.

4. A plastic hood to cover the trough and framework.

When the apparatus is used the electrophoresis papers, wetted with a mixture of 90 per cent methanol and 10 per cent acetic acid, are fastened to the framework as shown in Fig. 2. The trough is filled with the staining solution so that the liquid surface is about 1 cm above the lowest position of the paper strips. A rotation time of 20 minutes will usually be sufficient when a saturated solution of Amidoblack 10 B in methanol is used as the staining medium. The trough is emptied and refilled with the decoloring solution (for example 30 per cent acetone in water). In this case treatment in two successive baths, 15 min. in each bath, will usually give a clear white background.

Such apparatuses have been used the last two years in this laboratory and have contributed considerably to simplifying the paper electrophoretic analyses of serum proteins.

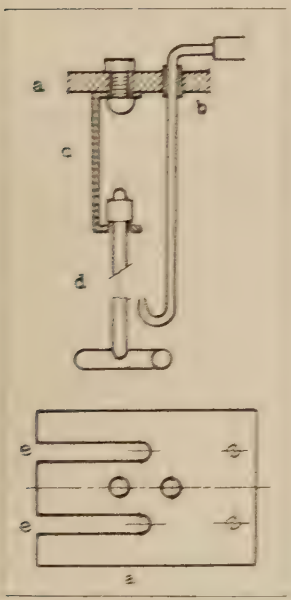
Scand. J. Clin. & Lab. Investigation 345, 10, 1958.

A DEVICE FOR MAGNETIC STIRRING IN ELECTROMETRIC TITRATION

By P. HANSEN

From The Central Laboratory Odense City and County Hospital Odense Denmark

Received for publication January 10, 1958.



1. Vertical and horizontal section of the stirrer.

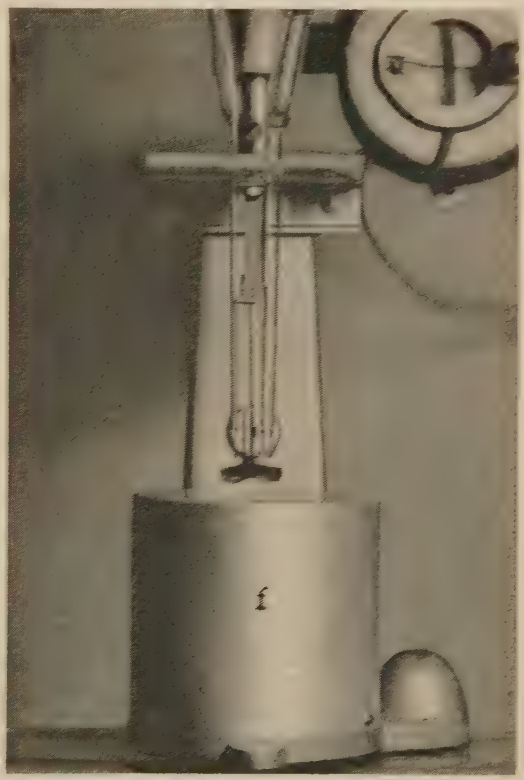


Fig. 2. The stirrer in its

or automatic electrometric titration we Radiometer Titration Assembly TTA 1 a magnetic stirring. As we did not find it by to move the agitators made as plastic-red magnets from one titration glass to ther, we made a stirrer suspended in a ing, where it remains during the chang- of the glasses.

ne drawing (Fig. 1) shows a plexiglass- tal, which is fixed to the titration ably. The two deep notches (e) are for electrodes. In a hole in the glass the for supplying titration fluid is fixed ttle by a small rubber dram (b). It ther holds the upper end of a double angled narrow steel plate (c) is fixed. In the lower end there is a hole serving as the bearing for the upper end of the stirrer.

The stirrer itself is shaped like an inverted T made of plastic (d). In the lower horizontal part a piece of iron is enclosed, which will make the stirrer rotate synchronously with the motor-driven magnet in the rotating table of the assembly (f in Fig. 2).

Letters to the Editors

Scandinav. J. Clin. & Lab. Investigation 346—347, 10, 1958.

METABOLIC CHANGES IN ACID-BASE EQUILIBRIUM DURING OPERATION AND ANESTHESIA

The fact that glucosuria and ketonuria arise during operations and anesthesia has been known for a long time.

The department of anesthesia at the Finsen Institute, Copenhagen, have ascertained pH, pCO_2 and $stHCO_3^-$ in the arterial blood under operations and anesthesia by means of Astrup's (1956) technique, which makes it possible to distinguish between metabolically conditioned changes in the acid-base status, expressed by standard bicarbonate ($stHCO_3^-$) (Jørgensen & Astrup, 1957) and respiratorially conditioned changes, expressed by pCO_2.

The 22 patients who were examined were all anesthetized by non-rebreathing technique and general analgesia, either with 75—80 per cent nitrous oxide or with 1.5 per cent ether in the inspired air. Respiration was in all cases artificial, and was of such a magnitude that the pCO_2 content in the arterial blood was between 20 and 25 mm Hk.

Fifteen of the 22 patients developed a non-respiratorially conditioned acidosis, which appeared to increase in accordance with the length of the operation. (Fig. 1.) Some of these patients were examined for and were found to have an increasing hyperglycemia and ketonuria.

The spontaneous alveolar ventilation was greater postoperatively than immediately before the operation, which is assumed to be due to a compensation for the metabolic acidosis developed during anesthesia.

Since the blood changes ascertained resembled those found in patients suffering from diabetes mellitus, an attempt was made in three cases to prevent the development of these changes or to treat them with insulin and glucose administered intravenously during the operation.

In the first case the content of standard bicarbonate was thereby kept more or less constant throughout an 8-hour operation

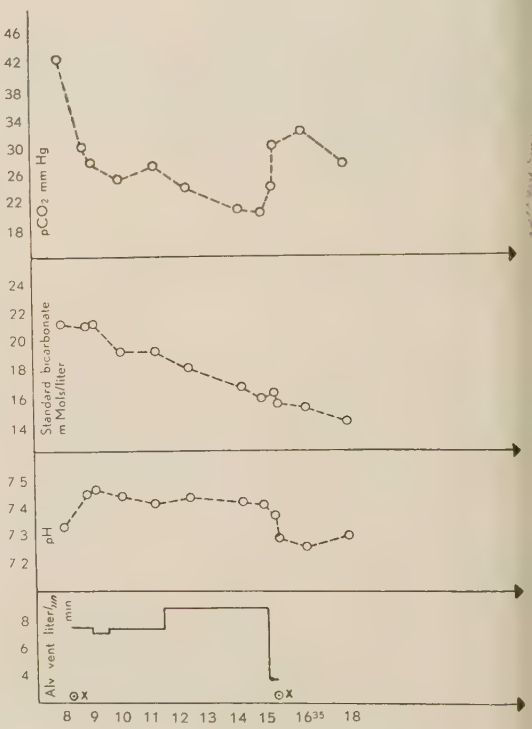


Fig. 1.

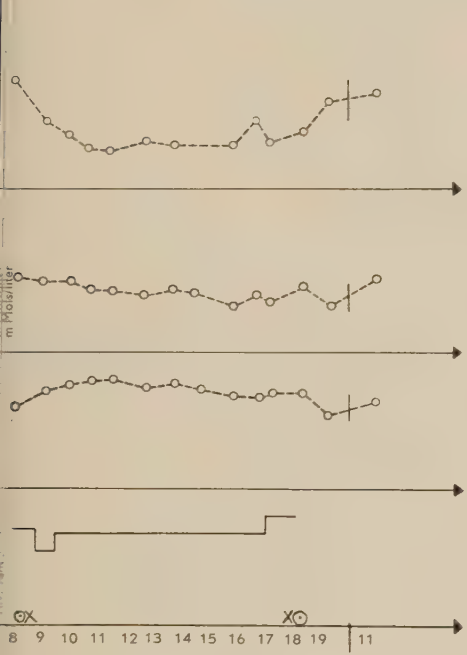


Fig. 2.

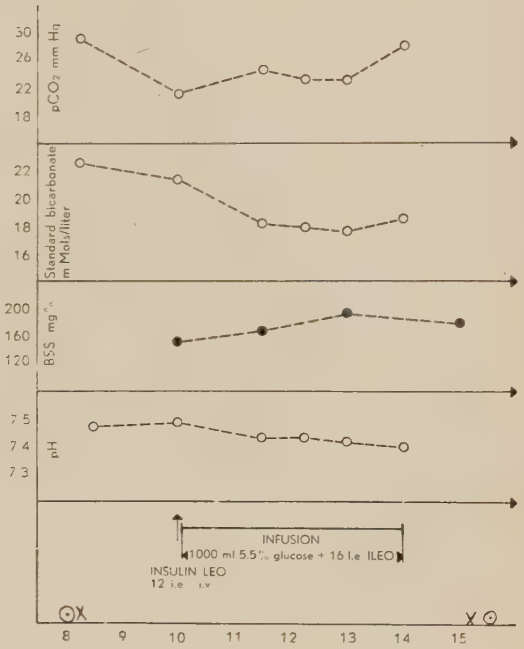


Fig. 3.

Fig. 2). In the second case, treatment first gan after a fall in the standard bicarbonate ntent had been ascertained. The fall was opped and changed to a slight rise (Fig. 3). he third case concerned a diabetic patient, whom the acid-base status was normalized uring operation.

Of the remaining four patients, the first owed no changes under a short exploratory oracotomy. The second patient, who was erated for a pancreatic stone, showed only slight fall in standard bicarbonate in spite the operation lasting 10 hours. The last wo, who were both icteric, both showed a ght rise in standard bicarbonate.

It is not believed that the metabolic aci-sis arises as a compensation for the hyper-ventilation undertaken during the anesthesia, since the titrimetrically determined quantity of acid excreted with the urine (Jørgensen, 1957) increased during the anesthesia at the same time as the content of standard bicarbonate fell.

Further investigations are in progress.

epartment of Anesthesia,
nsen Institute,
openhagen, Denmark.
pril 24, 1958.

REFERENCES

Astrup, P. & Schrøder, S.: A simple electrometric technique for the determination of carbon dioxide tension in blood plasma, total content of carbon dioxide in plasma, and bicarbonate content in "separated" plasma at a fixed carbon dioxide tension (40 mm Hg) Scandinav. J. Clin. Lab. Investigation *8*, 33, 1956.

Jørgensen, K.: Titrimetric determination of the net excretion of acid/base in urine. Scandinav. J. Clin. & Lab. Investigation *9*, 287, 1957.

— & Astrup, P.: Standard bicarbonate, its clinical significance and a new method for its determination. Scandinav. J. Clin. Lab. Investigation *9*, 122, 1957.

Erik Nandrup.

ELECTROPHORETIC SEPARATION OF SERUM PROTEIN INTO TWELVE FRACTIONS

Recently it has been demonstrated that the electrophoretic separation of serum proteins can be improved. Thus an increase in the number of separated electrophoretic components, or zones, from the conventional six to nine was made possible by the introduction of a new buffer (TRIS-EDTA-boric acid), the TEB-buffer (Aronsson & Grönwall, 1957).

We have now found that the number of separate zones obtained from a normal serum with this buffer on cellulose acetate strips (Oxoid) (Kohn, 1957) can be increased further through some minor modifications of the method. The concentration of the buffer has been reduced in relation to the buffer used in earlier experiments with cellulose acetate paper and the buffer now has the following composition:

TRIS	60.5 g/l
EDTA	6.0 g/l
Boric acid	4.6 g/l

pH 8.9; conductivity 3.0 mMho

The strips are 30 cm long which allows about 25 cm migration of the albumin front. The running time is 20 hours, the voltage gradient 6.0 V/cm. Staining with Amido Black 10 B.

Under these conditions a normal serum regularly will be separated into twelve fractions or zones. An example is given in Fig. 1. A study of how the new components are related to the conventional electrophoretic components is in progress. For the time being we have interpreted the separation in the following way.

Albumin separates into two fractions, prealbumin and one main fraction. α_1-globulin is split into two fractions and α_2 is split into three. The β-fractions are four altogether,

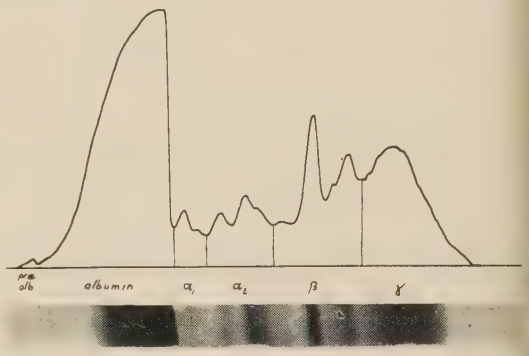


Fig. 1.

while the γ-globulin shows no obvious signs of splitting.

The recording densitometer "Analytrol" (Spinco Division, Belmont, California) has been used for evaluation of the strips. We have noticed that some components do not "come out" on the diagram as they do on the strip itself. This may partly be explained by the fact that the zones sometimes are curved or irregular and that the sensitivity and resolving power of the recording instrument is insufficient to register some of the smallest components. The result of an electrophoretic experiment is thus best evaluated by a combined examination of the diagram as well as of the stained strip (see Fig. 1).

The findings reported above show that the resolving power of zone electrophoresis can be improved by proper selection of the experimental conditions.

Department of Clinical Chemistry,
University Hospital,
Uppsala, Sweden.

May, 1958.

REFERENCES

Aronsson, T. & Grönwall, A.: Improved separation of serum proteins in paper electrophoresis — a new electrophoresis buffer. Scandinav. J. Clin. Lab. Investigation *9*, 338, 1957.

Kohn, J.: A cellulose acetate supporting medium for zone electrophoresis. Clin. Chim. Acta *2*, 297, 1957.

T. Aronsson,
A. Grönwall.

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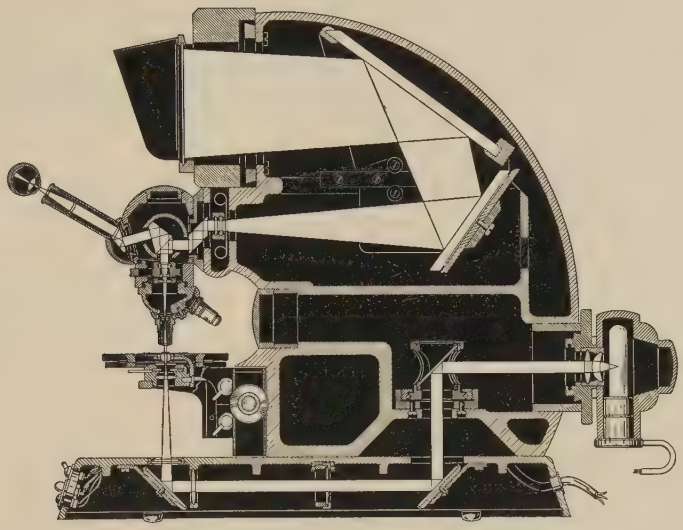

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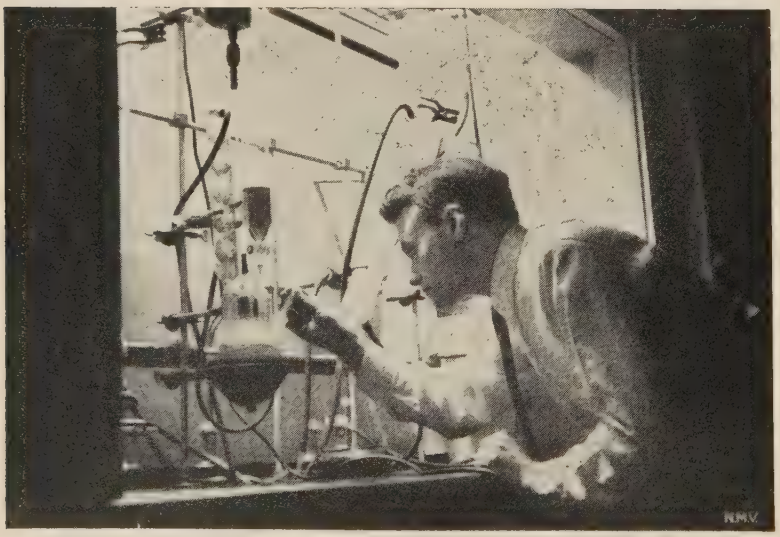

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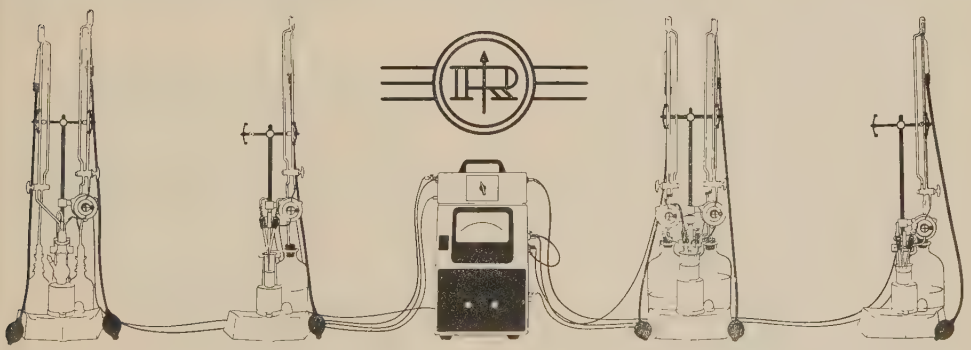

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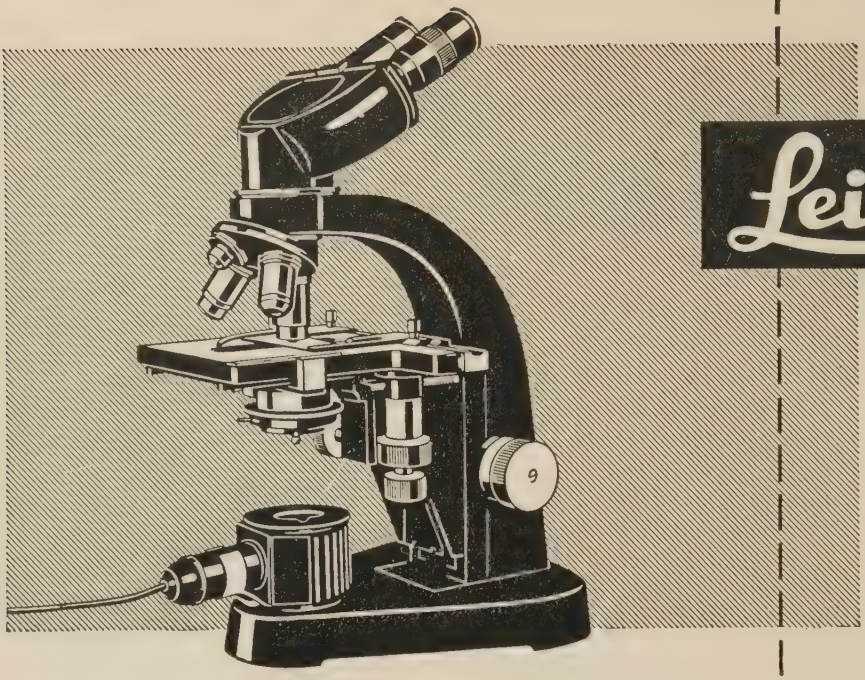

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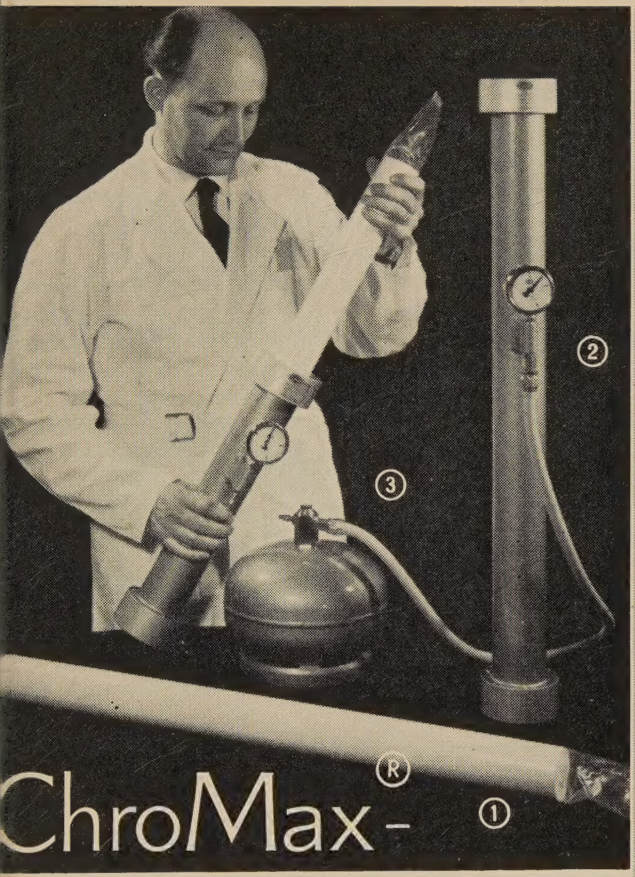

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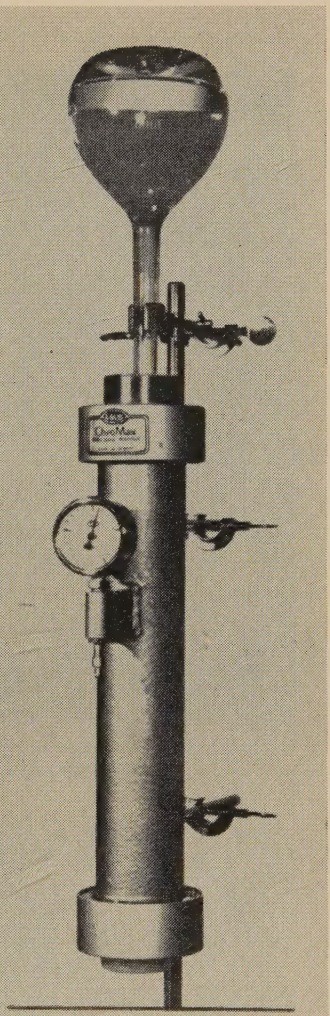

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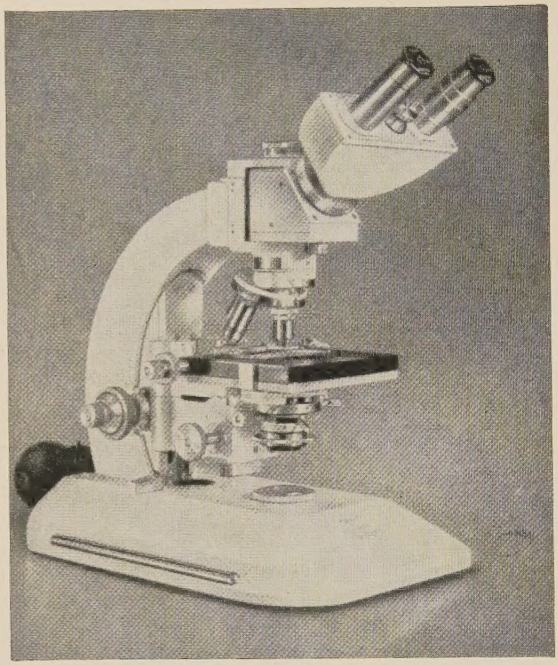

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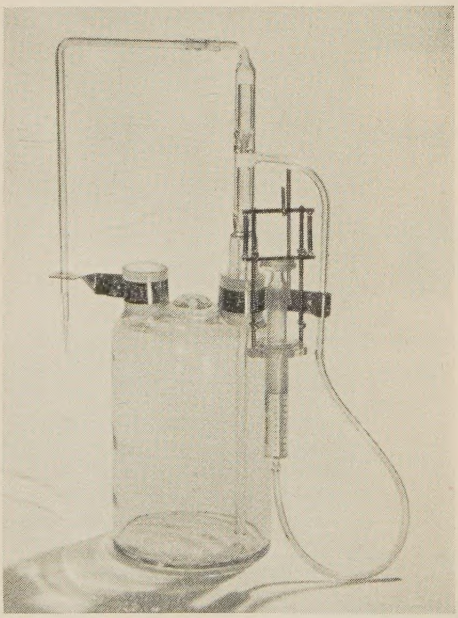

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